Regulation of Ion Channel Physiology in Airway Epithelial cells in response to Influenza A Virus infection

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By

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Abstract

Epithelial cells lining the upper airways are characterized by low sodium absorption and elevated chloride secretion. Together, the movement of these ions creates the osmotic drive to hydrate the airways. Recent studies indicate that influenza is capable of directly modulating the vectorial transport of sodium and chloride ions. However, the direct impact of influenza has not been studied with respect to potassium channels. This is significant because potassium conductance creates the driving force for chloride secretion. Disruptions to this process leads to edema formation in the lungs and can subsequently cause Acute Respiratory Distress Syndrome. Additionally, it has been demonstrated that the induction of pro-inflammatory cytokines in infected cells may contribute to altered ion channel function, further exacerbating edema formation. The purpose of this study was to assess the direct and indirect effects of influenza virus infection on potassium and chloride ion channel function in a secretory epithelial cell model.

In order to assess the direct effects we exposed polarized epithelial cell monolayers to varying doses of H1N1 virus. Potassium and chloride channel function was measured by means of short-circuit current in an Ussing chamber. The immune response to viral infection was determined by RT-qPCR and Bioplex suspension array. Virus conditioned media (CM), and IL-8 were used to characterize the indirect effects on non-infected cells.

We observed an increase in chloride secretion, consistent with edema formation, when 60% of the epithelium was infected, and after CM treatment. This observation correlated with increased potassium channel conductance through the

calcium-activated (KCNN4) and cAMP-activated potassium channels (KCNQ1), which was ameliorated upon specific inhibition of these channels. The data suggest that the mixture of pro-inflammatory cytokines induced by viral infection directly up-regulate these potassium channels. However, treatment with IL-8 also appears to increase chloride secretion, although the underlying mechanism remains to be determined, as it is not mediated through KCNN4 and KCNQ1. We conclude that the strong induction of cytokines in infected cells act in a paracrine manner on non-infected cells to increase potassium channel conductance. This up-regulation of potassium channels subsequently drives an increase in chloride secretion, leading to fluid build-up in the lungs and edema formation.

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Dedication

I would like to dedicate this research to my great grandmother Lena Solberg who inspired me to follow my dreams despite all obstacles.

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List of Abbreviations

AQP – Aquaporin

ARDS – Acute Respiratory Distress Syndrome

ASL – Airway surface liquid

ATP1A1 – Na^+-K^+ ATPase

ATI – Alveolar Type 1 cells

ATII – Alveolar Type 2 cells

CaCC – Calcium-activated Chloride Channel

cAMP – Cyclic adenosine monophosphate

CCM – Control Conditioned Media

CFTR – Cystic Fibrosis Transmembrane Regulator

Cl^- – Chloride ion

ENaC – Epithelial Sodium Channel

I_{sc} – Short-circuit current

IFN – Interferon

IL – Interleukin

K^+ – Potassium ion

K2P – Two-pore K^+ channel

KCNN4 – Ca^{2+}-activated K^+ channel

KCNQ1 – cAMP-activated K^+ channel

MOI – Multiplicity of Infection

Na^+ – Sodium ion

Nedd4 – Neuroneal precursor cells expressed developmentally down-regulated 4

NF-kB – Nuclear factor kappa beta

NHBE – Normal Human Bronchial Epithelial cell

NKCC1 – Na^+-K^+-$2Cl^-$ Co-transporter

PAMP – Pathogen-associated molecular patterns

PFU – Plaque-forming unit

PKC – Protein Kinase C

PRR – Pattern recognition receptor

RANTES – Regulated on Activation, Normal T cell Expressed and Secreted

RLR – RIG-I-like receptor

RNA – Ribonucleic acid

RSV – Respiratory Syncytial Virus

TLR – Toll-like receptor

TNF – Tumor necrosis factor

VCM – Virus Conditioned Media

CHAPTER 1. LITERATURE REVIEW

1.1. Background

1.1.1. Influenza A virus

Influenza A virus is part of the *Orthomyxoviridae* family of negative-stranded RNA viruses[1]. The viral genome is comprised of eight segments, which code for 11 different proteins[2]. Two of these proteins, hemagglutinin (HA) and neuraminidase (NA), are found on the viral envelope; differences among these proteins allow for classification of influenza subtypes (e.g., H1N1, H5N1, etc)[3]. H1N1, in particular, has been widely studied, as it has been the cause of at least two worldwide pandemics since the early 1900's[1]. Influenza A viruses can infect, and transmit between, a wide variety of hosts; human, swine and avian hosts are most common[3,4]. In nature, multiple strains of influenza virus can infect the same host[5]. When this occurs, the genomes of the different viruses can mix, causing dramatic changes to the surface glycoproteins[1,5,6]. This leads to the formation of pandemic strains and is defined as antigenic shift[2,7]. Alternatively, seasonal strains of influenza arise from a process termed antigenic drift, which refers to minor changes in the surface glycoproteins that arise due to mutation[6,7]. Efforts to protect individuals against influenza infection have been largely unsuccessful in the long term because of the ability of the virus to undergo both antigenic drift and antigenic shift[5]. Since these processes occur randomly, and the mutation rate is also influenced by the host immune system, it is difficult to consistently predict the

annual circulating strains[8,9]. This poses a significant challenge to researchers in the development of vaccines[8]. Therefore, for effective management of infected patients an understanding of the pulmonary pathophysiology associated with influenza infection is required.

1.1.2. Overview of airway epithelium

The entire respiratory tract is lined with epithelial cells, which provide a physical barrier to inhaled environmental pathogens[10,11]. The epithelial cells form tight junctions, dividing the cells into apical (airway-facing) and basolateral (blood-facing) surfaces[10]. In general, ion channels localized to either the apical or basolateral surface of these cells work in concert to move solutes, creating the osmotic drive to move water into or out of the airways[11]. This entire process is driven by the basolaterally located $3Na^+/2K^+$-ATPase pump, which pumps out three sodium ions (Na^+) in exchange for two potassium ions (K^+)[11]. This creates the electrochemical gradient that drives the passive "leakage" of sodium, chloride, and potassium through ion channels[11,12]. Maintenance of a healthy lung is dependent on these ion transport processes, as they generate the osmotic drive to hydrate the airways[12]. This pump-leak model of ion transport regulates the fluid volume in the lung, maintaining it at appropriate levels to ultimately facilitate proper gas exchange[11,13]. This model outlines the basic driving force for the movement of solutes and water in the airways. However, ion channel distribution differs between cell types, changing the cellular or tissue phenotype. Cell type distribution varies between the upper and lower respiratory tract (detailed below); it is this variation

that partially determines the secretory or absorptive characteristics of the upper and lower airways, respectively[14].

1.1.1.1. Upper respiratory tract

The upper respiratory tract is comprised of the nasal cavity, and trachea[11]. Epithelial cells lining the upper airways form the surface epithelium, which contains a number of different cell types that contribute to airway hydration[11]. In addition, submucosal glands underlying the surface epithelium contribute to fluid secretion[14,15,16]. The majority of the fluid is secreted by serous cells, which predominate the acinus (base) of the submucosal glands[14,15]. Movement of fluid through the glands subsequently pushes out mucus generated by mucus cells located further up the duct[14,17]. This fluid, now termed periciliary fluid, supports the mucus layer and together these form the airway surface liquid (ASL)[11,15]. The ASL is maintained through both anion and cation transport processes across the epithelium, which is essential for mucociliary clearance[11,12,18].

The ciliary beat, which facilitates mucociliary clearance, is dependent on appropriate periciliary fluid volume, which elevates the mucus above the cilia[14,18]. The mucus layer traps debris and is subsequently removed from the airways[19]. Hydration of the mucus also reduces the viscosity, facilitating mucociliary clearance[20]. Overall, this process is a large part of the innate immune system in the lungs[18,19].

The ion transport processes that drive ASL formation are outlined in Figure 1. The electrochemical gradient that drives fluid secretion is maintained through the active pumping action of the basolateral Na^+/K^+-ATPase, which exchanges $3Na^+$ for

$2K^+$[12]. The stoichiometry creates a charge separation resulting in a high internal potassium concentration and low internal Na^+[21]. Naturally, Na^+ follows its electrochemical gradient and moves into the cell via the apical epithelial sodium channel (ENaC)[12,22]. The efflux of K^+ from the apical and/or basolateral membranes, facilitated through K^+ channels (e.g., KCNN4, KCNQ1), increases the negative change within the cell, hyperpolarizing the membrane, and driving chloride (Cl^-) secretion[11,12,23]. In the apical membrane, Cl^- moves through two different channels: the cAMP-activated cystic fibrosis transmembrane regulator (CFTR) channel, and the calcium-activated chloride channel (CaCC)[11]. TMEM16A is the primary CaCC present in airway epithelium; however, this channel accounts for a smaller proportion of the overall chloride conductance in comparison to CFTR[24,25]. The intracellular stores of Cl^- are replenished by the electroneutral intake of Na^+, K^+, and $2Cl^-$ on the basolateral side via NKCC1[11]. In the upper airways, CFTR is highly abundant and acts to down-regulate ENaC function, leading to reduced Na^+ absorption across the apical membrane[26,27]. This combined effect of Na^+ and Cl^- transport generates a strong osmotic drive for hydrating the upper airways. Water then follows this osmotic gradient, moving either paracellularly, or through apical and basolateral aquaporin channels (AQP5, 3, 4), hydrating the airways[20,28].

1.1.1.2. Lower respiratory tract

The lower respiratory tract is made up of the bronchi and alveoli[11]. The bronchi are covered by surface epithelium, and is aglandular[15]. The alveolar

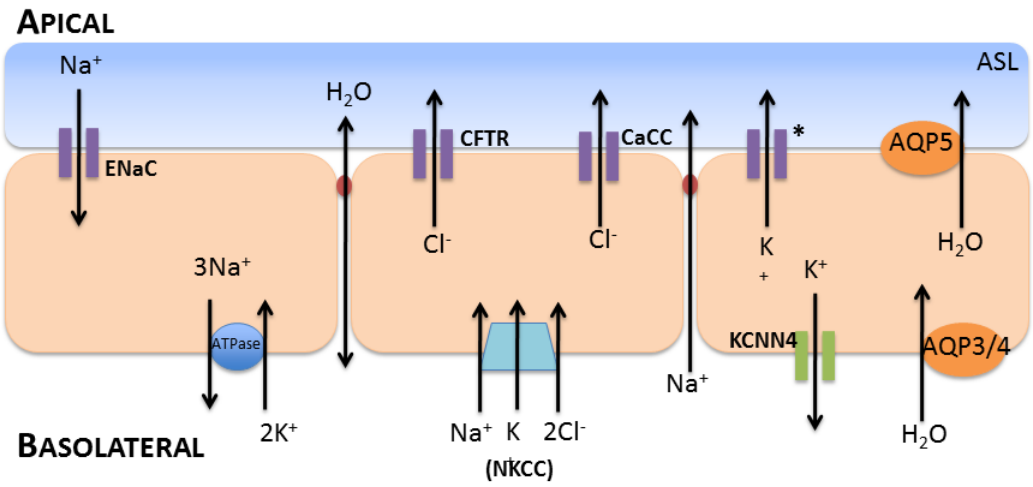

Figure 1. Overview of upper respiratory tract ion transport processes.

Represented are the Na^+, Cl^-, and K^+ channels, along with their respective transporters. This diagram represents the channels in different cells for simplicity. The apical side corresponds to the airspace, where the ASL is secreted. Submucosal gland serous cell secretions create the osmotic drive to generate the ASL (see text for details). Upper respiratory cells are characteristically secretory in order to maintain appropriate ASL volumes to facilitate mucociliary clearance. ENaC = epithelial sodium channel, comprised of three subunits (α, β, and γ). CFTR = cystic fibrosis transmembrane regulator channel. CaCC = calcium-activated chloride channel (TMEM16A). AQP = aquaporin (H_2O) channels. KCNN4 = calcium-activated potassium channel. * = Apical potassium channel; varies between cell type. NKCC = $Na^+/K^+/2Cl^-$ co-transporter. ATPase = Na^+/K^+-ATPase.

epithelium is made up of two different cell types, alveolar type I (ATI) and type II (ATII) cells. ATI cells are large and squamous, comprising over 95% of the epithelial surface[29]. Their main function is gas exchange; however, there is evidence supporting their role in maintaining lung fluid homeostasis through both ion and water transport (Figure 2)[30,31]. These cells are extremely permeable to water, as they express high levels of aquaporin 5 (AQP5) in their apical membrane[30,32]. More recent evidence has indicated that ATI cells contain several cation channels both amiloride-sensitive (ENaC), and –insensitive channels, and the basolateral Na^+/K^+-ATPase[30,31,33]. This basolateral pump creates the electrochemical gradient for the apical absorption of Na^+[30]. Apical chloride (Cl^-) channels, namely CFTR, facilitate the movement of Cl^- in order to maintain electroneutrality[33]. Additionally, evidence suggests that both ATI and ATII cells contain potassium (K^+) channels in the apical membrane[34]. The outward movement of K^+ further drives fluid absorption in the lower airways[30]. Overall, the low levels of CFTR and high abundance of Na^+ channels relative to the upper airways promote an absorptive phenotype[30,32].

ATII cells are much smaller, and make up the remaining 5% of the alveolar epithelial surface[11]. They secrete surfactants, which are important for reducing surface tension, and promoting gas exchange[11,35]. Similar to the ATI cells, the basolateral membrane contains a Na^+/K^+-ATPase responsible for driving the net absorption of fluid, making the driving forces for fluid absorption in ATII cells nearly identical to those in ATI cells[30,31]. The main difference observed between ATI and ATII cells is the lack of aquaporins in ATII cells (Figure 2)[32,36]. Despite this

difference, the net movement of fluid in the lower airways remains apical to basolateral, as a result of the vectorial transport of Na^+ and Cl^-. This creates the osmotic drive to move water across the apical membrane.

This vectorial transport in the lower respiratory tract is made possible by the presence of both ENaC and CFTR[22,30,37,38]. Although the roles for both of these ion channels have been widely studied, there is no consensus on whether alveolar cells generally act in a secretory or absorptive manner[11,29,30,36,39]. Since many different models have been employed to study the lower airways (see "*Models for studying chloride conductance in the lower airway*"), it has been difficult to accurately determine the phenotype as being either secretory or absorptive. However, we know that hydrostatic pressure forces fluid into the airways, and that the upper airways secrete fluid to form the ASL[39,40]. Additionally, there is a growing body of evidence surrounding the presence of multiple different sodium channels in the alveolar epithelium, which would increase the absorptive capacity[30,32,40]. Taken together, it would seem reasonable to conclude that, in general, the lower respiratory tract has the capacity and the need to absorb fluid. However, there is evidence to support both absorptive and secretory characteristics to maintain lung liquid homeostasis.

1.1.3. Epithelial immune response to influenza virus infection

Influenza viruses primarily attach to, and infect, ciliated epithelial cells of the upper respiratory tract[7,35]. The viral hemagglutinin binds to sialic acid residues, arranged either in the α2,6- or α2,3 linkage[41].

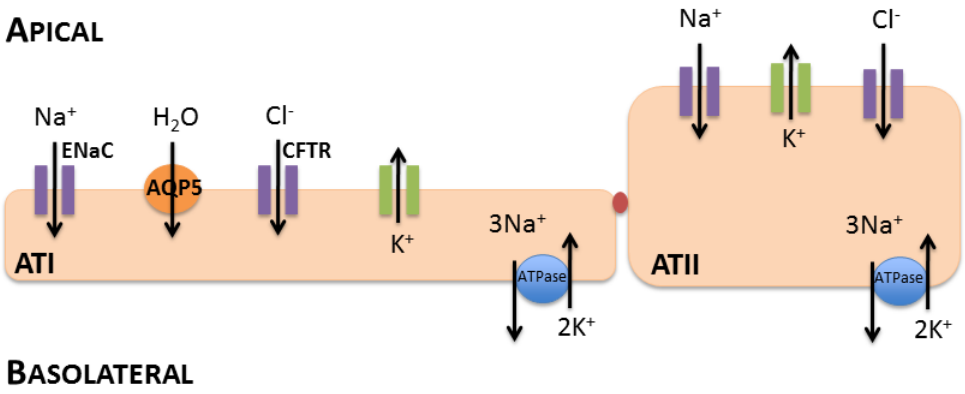

Figure 2. Overview of the lower respiratory tract ion transport processes

ATI and ATII cells play a role in vectorial ion transport driven through the Na^+/K^+-ATPase (see text for details). ATI cells are responsible for transporting a large portion of H_2O via aquaporin 5 in the apical membrane. The presence of Na^+, Cl^-, and K^+ channels in both cells is evidence that the alveolar epithelium, in general, is responsible for helping maintain lung liquid homeostasis. ATI = alveolar type I cells. ATII = alveolar type II cells.

The affinity of hemagglutinin for a particular form of sialic acid is highly dependent on the species that the virus naturally infects (e.g., human virus isolates tend to prefer the α2,6 linkage)[42,43]. The α2,6 linkage is abundant in the upper airways, making this the primary site of infection for strains such as H1N1[41]. However, epithelial cells in the lower airways are also permissive to influenza infection[7,35]. They become infected as a result of increased viral load[35]. This movement of the virus into the lower airways results in complications, such as viral pneumonia[35,44]. ATI and ATII cells work together to clear excess fluid from the airspace (see above, "*Lower respiratory tract").* This ability is severely compromised during influenza virus infection, and results in flooding of fluid into the airspace[44]. The main consequence is poor gas exchange, due to the development of pulmonary edema[7,23]. This edema formation is partly driven by changes in the epithelium induced by both the virus and the host innate immune response[45]. This immune response is two-fold and includes both resident alveolar macrophages present in the lung, as well as the epithelial cells themselves[44].

Resident alveolar macrophages present in the lung are the first immune cells to encounter inhaled pathogens[46]. Encountering a pathogen activates these cells, which then secrete various cytokines and chemokines in order to stimulate the innate immune system. The most common pro-inflammatory cytokine produced by macrophages is tumor necrosis factor-α (TNF-α)[46,47]. TNF-α is an important immune mediator, and has also been shown to impact sodium ion transport through down-regulation of ENaC (see "*Role of pro-inflammatory cytokines*")[48]. These secreted products drive an inflammatory response by recruiting dendritic cells,

natural killer cells, neutrophils, and T lymphocytes to the site of infection[46,49].

The epithelial cells also have the capacity to contribute to the inflammatory response after viral infection[10,19,50,51]. Recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) such as toll-like receptor (TLR) 3 and TLR7, as well as the retinoic acid inducible gene-I (RIG-I), results in the release of cytokines and chemokines from epithelial cells (see "*Activation of the innate immune system*")[10,52,53]. The production of pro-inflammatory cytokines (e.g., IL-6, IFN-γ, IL-8, IL-1β, etc) aids in promoting an antiviral state, and further recruits immune cells[54,55]. However, this strong pro-inflammatory cytokine response elicited from both macrophages and the epithelium has been implicated in the development of ARDS[46,56]. This syndrome, which can result from infection, or any traumatic injury to the lung, is driven by the release of a cytokine cascade[57]. The inflammatory response associated with ARDS likely contributes to the formation of pulmonary edema by disrupting the vectorial ion transport. More specifically, it is likely the cytokines, which have been shown to modify ion transport, that drive this pulmonary edema formation (see "*Role of pro-inflammatory cytokines*")[48,58,59].

The strong inflammatory cytokine release responsible for ARDS, and the resulting pulmonary edema, has not been properly investigated in a secretory model. Currently, studies looking at the direct effect of influenza infection on a secretory model are very limited, as most studies have been focused on the inhibition of absorption[60,61,62]. It is for these reasons we chose a secretory cell model, Calu3, to study the effects of the virus, as well as the immune response on

fluid secretion. The Calu3 cell line is derived from a human lung adenocarcinoma, and has been well characterized in the literature as a useful model for studying submucosal gland serous cells[63,64,65]. These cells have been shown to be permissive to both human and avian strains of influenza, making it an excellent model to study the effects of the virus[4,66,67]. Additionally, Calu3 cells form polarized monolayers with tight junctions in culture; these monolayers allow for the study of transepithelial ion transport, as measured by short-circuit current in an Ussing chamber[63,68]. They also express high levels of CFTR, and have been shown to contain basolateral potassium channels[64,65]. These potassium channels play a key role in driving apical anion secretion[65,69]. This immortal cell line provides a dependable, convenient and reproducible model for studying the interactions between virus and host with respect to ion transport, and cytokine response[66].

1.2. Direct effects of influenza on epithelial cell ion channel physiology

1.2.1. Sodium absorption

The mechanisms driving Na^+ absorption across the apical membrane have been extensively studied[27,70,71,72]. A study done by Hummler *et al*., identified that the α-subunit of the ENaC is critical immediately after birth in clearing fluid from the lungs[72]. The inability to facilitate the removal of fluid from the lungs of αENaC-deficient mice resulted in death, due to respiratory failure[72]. These data would imply that the α-subunit plays a pivotal role in airway epithelial Na^+ absorption, especially in the infant lung. However, in the adult lung, a balance between both absorptive and secretory processes is required in order to maintain

ASL levels[62,70]. Disruptions to the fluid balance, such as during influenza infection, lead to a reduced ability to clear fluid from the lungs.

Influenza virus has been shown in the initial stages of infection to inhibit sodium absorption through modulation of ENaC[60,62]. Kunzelmann *et al*. exposed epithelial cells to UV-inactivated influenza virus (A/PR/8/34 H1N1) for 1 hour and showed no effect on the amiloride-sensitive short-circuit current, indicating that the inactive viral proteins do not lead to any downstream impacts on Na^+ absorption through ENaC[60]. This phenomenon contradicts their conclusions from subsequent experiments that indicate detergent-disrupted virus was able to inhibit Na^+ absorption[60]. Overall, their final conclusion is that hemagglutinin binding to its cellular receptor leads to a decrease in Na^+ reabsorption, and thus reduced fluid clearance from the lungs[60]. In addition to this, Chen *et al*. showed that influenza A virus infection reduces ENaC function via phospholipase C-induced activation of PKC. This occurred after binding of hemagglutinin to the cell surface (Figure 3)[62]. Moreover, their work distinguishes that the observed reduction in fluid clearance is a result of decreased Na^+ channels, and not a result of increased epithelium permeability[62]. This distinction is important because it aids in the understanding that respiratory viruses, such as influenza, have physiological impacts that supersede the cytopathic effects that can eventually compromise the epithelial barrier resulting in increased fluid permeability. Studies done *in vivo* by Wolk *et al*., provide corroborating evidence to support the role that PKC plays in ENaC down-regulation, and subsequent reduction in sodium reabsorption[61].

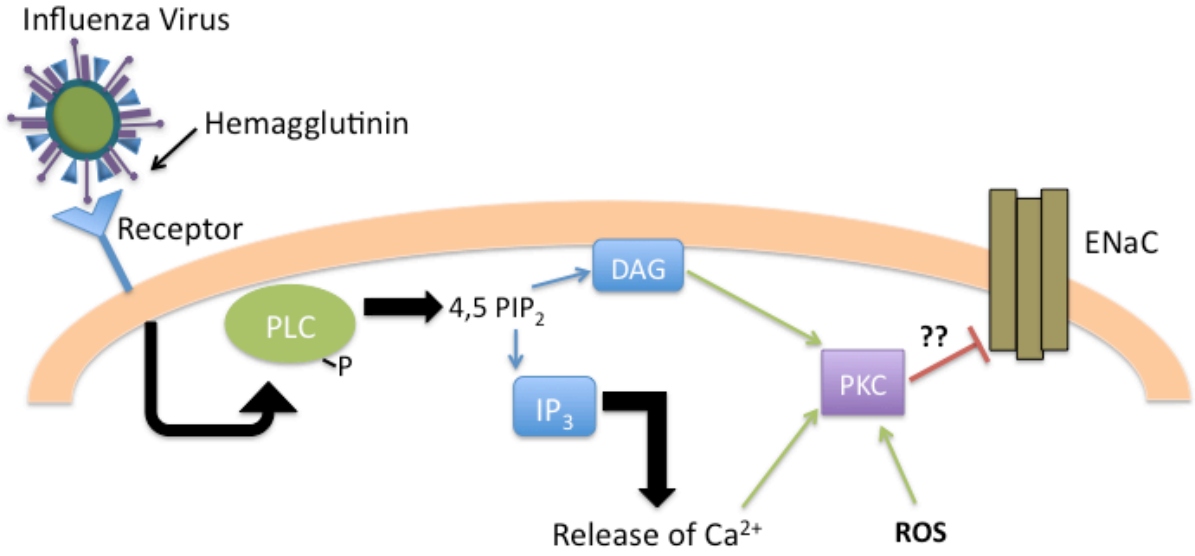

Figure 3. Effects of activated protein kinase C (PKC) on epithelial sodium channel (ENaC) expression.

The initial stages of influenza virus infection involve binding of the hemagglutinin to sialic acid residues on the host cell. Down regulation of ENaC has been detected as early as 1 hour post-infection with influenza. Binding of hemagglutinin stimulates the phosphorylation of phospholipase C (PLC). Once phosphorylated, PLC cleaves the precursor phopholipid phosphatidylinositol 4,5 biphosphate (4,5 PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 binds to downstream receptors to release intracellular stores of calcium (Ca^{2+}). The release of Ca^{2+}, along with DAG work together to activate PKC. Reactive oxygen species (ROS) have also been implicated in activating PKC. Activated PKC acts to inhibit ENaC through an unknown mechanism. This inhibition results in decreased Na^+ reabsorption across the apical membrane.

1.2.1.1. Involvement of protein kinase C

Much emphasis has been placed on the regulation of ENaC through protein kinase C (PKC)[60,62,70,73,74]. PKC has been shown to inhibit ENaC in the airways, specifically targeting the α-subunit[62,74,75]. In addition, PKC has also been shown to inhibit ENaC in renal tissue through inhibition of the β- and γ-subunits[27,76]. In the kidneys, these subunits play a larger role in ion transport than the α-subunit[27]. Clearly, PKC activation has varied effects on ENaC in different tissues[74]. Furthermore, it appears that influenza virus replication is actually enhanced in cells that are treated with sodium channel inhibitors, or activators of PKC[77]. In contrast, inhibiting PKC and/or stimulating sodium channels *in vitro* reduces influenza's ability to enter cells and replicate[77,78]. Hoffmann *et al*. screened for compounds that may impact influenza virus growth, and identified that the virus is sensitive to fluctuations in intracellular ion concentrations; therefore, the down-regulation of ENaC may act to facilitate viral growth and proliferation[75]. Given this, it would seem reasonable to assume that, over time, the virus has evolved this strategy to modulate ENaC in order to ensure its success in replicating. Further investigation using the viral protein, M2, has shown that there is an increase in reactive species (oxygen and nitrogen), which act to down-regulate ENaC levels through activation of PKC[76]. Although the exact mechanism of how PKC acts to down-regulate ENaC activity is not fully understood, there are a number of competing hypotheses[76].

It is known that ENaC contains certain domains responsible for binding regulatory proteins[79]. One of these proteins, Nedd4 (neuroneal precursor cells

expressed developmentally down-regulated 4), is an ubiquitin protein that has been shown to have regulatory effects on ENaC[79,80]. Elevation of Nedd4 protein is controlled by release of intracellular Ca^{2+} stores, and this increase will lead to ubiquitination and subsequent degradation of ENaC (Figure 4)[79]. Alternatively, as proposed by Wolk *et al*., stimulation of purinergic receptor, P2Y, by UTP will act to decrease Na^+ absorption by decreasing ENaC activity[61]. However, this pathway also appears to require the activation of PKC[76,81]. Taken together, these studies display a role PKC activation plays in modulating ENaC levels in epithelial cells. More specifically, PKC is connected to the modulation of Na^+ transport resulting in a reduced ability to clear fluid from the lungs.

Interestingly, Palmer-Densmore *et al*. indicated that stimulation of epithelial cells with UTP increased Cl^- secretion, which may be through CaCC since UTP causes an increase in intracellular calcium[81]. In addition, PKC is capable of activating chloride channels (CFTR), which also has implications in the pathogenesis of influenza virus infections. This reciprocal activation of chloride secretion and decreased sodium absorption via calcium/PKC pathways ultimately results in fluid secretion into the lungs.

1.2.2. Chloride conductance

The role that Cl^- conductance plays in response to influenza virus infection is less well defined than the role of Na^+. It is known that both CFTR and CaCCs (TMEM16A) play an important role in driving apical Cl^- secretion in the upper respiratory tract[24,25,82]. This process, in concert with Na^+ absorption, generates

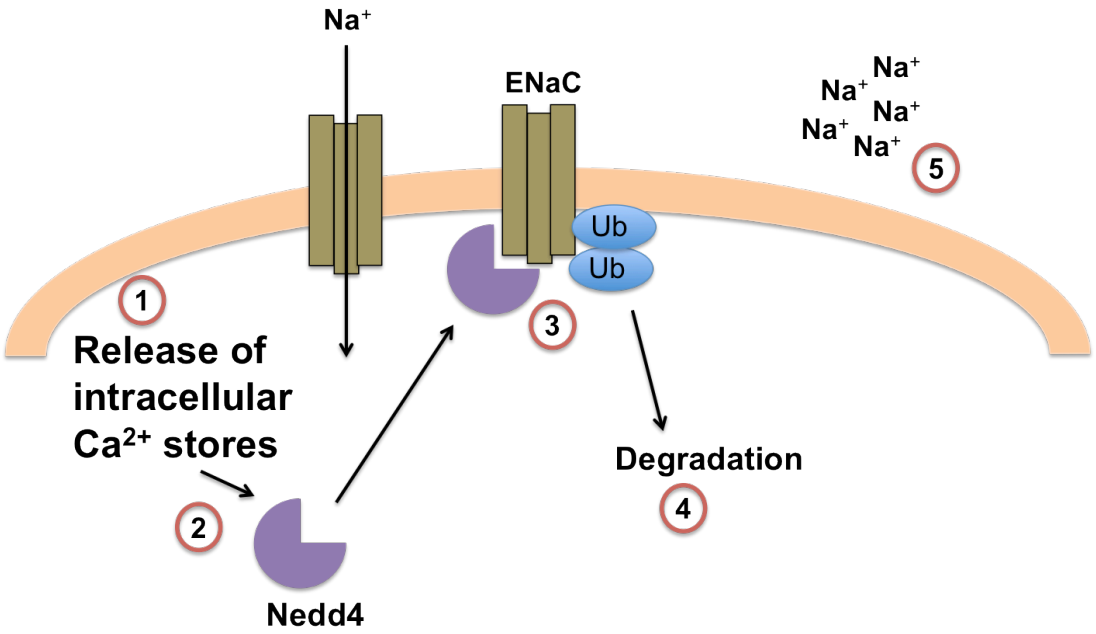

Figure 4. Overview of ENaC degradation pathway by Nedd4.

Schematic diagram of how ENaC is targeted for degradation by Nedd4 (neuroneal precursor cells expressed developmentally down-regulated 4). 1) Intracellular calcium stores are released. This is mediated by influenza binding sialic acid and subsequently phosphorylates PLC. PLC activation results in the internal release of calcium through IP_3 (see Figure 5A). This leads to increased levels of Nedd4. 2) Nedd4, an ubiquitin protein, is found in the cytosol. It is translocated to the apical membrane. 3) Binding domains found on ENaC bind Nedd4, which leads to ubiquitination (Ub) of the ENaC subunits. 4) This ubiquitination leads to endocytosis and subsequent degradation of ENaC. 5) A reduction in the number of ENaCs results in a reduced ability to absorb Na^+ across the apical membrane.

the transepithelial osmotic gradient that drives water across the epithelial barrier (see "*Overview of airway epithelium*" above).

In vitro models, such as Calu3 and normal human bronchial epithelial cells (NHBE), have been used to study chloride conductance in the upper airways[63,64]. Calu3 cells, in particular, are often chosen over NHBE cells, as they are well characterized as an anion secretion model[63,67]. Additionally, NHBE cells are a primary cell line that is more difficult to culture (relative to Calu3), and depending on culture conditions they may express different cellular phenotypes[67,83,84]. In the lower respiratory tract, the movement of chloride either into or out of the cell will play a large role in determining whether the epithelium is absorptive or secretory[11,22,85]. More recent evidence suggests that the lower airways are more absorptive; however, differences exist between the models used to study the lower airways. This is likely why there is still varying interpretation over the exact role chloride plays in either absorptive or secretory capacity in the lower airways.

1.2.2.1. Models for studying chloride conductance in the upper airways

In the upper respiratory tract, both the surface epithelium and the submucosal glands act to secrete fluid into the airways (see "*Upper respiratory tract*" above). The Calu3 cell line is an ideal model for studying anion transport, as they endogenously express high levels of CFTR in the apical membrane[63,86]. A cellular model, as described by Devor *et al*., indicates that Calu3 cells also contain a basolateral Na^+-K^+-ATPase pump along with the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1)[64]. This relatively simple model is a useful tool for studying both cAMP- and calcium-activated anion transport, which can be measured by changes in

transepithelial short-circuit current[87,88]. Secretion through CFTR is increased by stimulation with cAMP[86,89]. Additionally, there are both cAMP- and calcium-activated potassium channels, which are responsible for establishing the driving force for apical chloride secretion via hyperpolarization of the cell (see "*Potassium conductance*" below)[65,89].

Chloride transport in the primary NHBE cell line is nearly identical to that of Calu3 cells[90,91]. Primary cell lines, such as NHBE, offer an advantage over immortal cell lines in that they are more biochemically similar to the original tissue[92]. However, they are more challenging to culture, and variations between donors cannot be accounted for properly[88,92]. Therefore, Calu3 cells remain a primary model for studying anion secretion[84].

1.2.2.2. Models for studying chloride conductance in the lower airways

The majority of studies performed on lower respiratory epithelial cells use primary cell culture models[84,92,93]. There are, of course, limitations to any *in vitro* study, given the nature of the model. However, the models for alveolar cells in particular have a great amount of diversity[94]. More specifically, work done by Brochiero *et al*. has shown that alveolar type II cells grown in an air-liquid interface (a technique used to polarize epithelial cells) express higher levels of CFTR compared to those grown on submerged supports[22]. Moreover, the methods used for removal and isolation of alveolar cells for primary culture vary, leading to considerable differences in cellular morphology[30,95]. This change in cellular phenotype is exacerbated over time in culture, as shown by both Brochiero *et al.,* and Dobbs *et al.[22,95].*

More recently, it has become evident that ATI cells do, in fact, play a role in ion transport processes[30,31]. Given that ATI cells dominate the cellular population in the lower respiratory tract, understanding their contribution to ion transport, and the movement of fluid, is crucial. However, the isolation techniques for ATI cells are less developed than those for ATII cells[23,29]. Consequently, this means that the majority of studies use ATII cells, or ATI-like cells (which are ATII cells which have been differentiated to express ATI phenotypic markers)[30,44]. This raises a number of issues given that ATI cells are now implicated in more complex transport processes. Hence, it remains unclear whether the use of a single cell type can generate data that can accurately infer distinct ATI and ATII physiology. Johnson *et al*. raise these same concerns, as there is a marked difference in gene expression between cultured ATII and ATI cells[30]. Thus, it is not surprising that the role of chloride transport in the alveoli remains unclear. However, identification of selective and non-selective apical Na^+ channels (see "*Lower respiratory tract"* above), and K^+ channels (see "*Potassium conductance*" below) would suggest that the alveolar epithelium is primarily absorptive[30,32,33].

Not all of the models that are currently used to study Cl^- transport require cell culture. Many studies take advantage of animal models (*Mus musculus*, *Rattus norvegicus*, and *Xenopus laevis* are most common), which allow researchers to look at the interactions between different ion channels (i.e., ENaC and CFTR)[61,94,96,97]. These types of interactions may be important in elucidating the underlying mechanisms that drive fluid clearance after viral infection. Moreover,

animal models provide a way to study longer-term effects of viral infection, which are not necessarily feasible *in vitro[61]*.

1.2.2.3. Secretion or absorption in the alveoli

As outlined above, the variability in cell models likely impacts the ability to accurately determine the role that chloride channels play in regulating fluid levels in the alveoli. Studies involving freshly isolated *Xenopus laevis* lung epithelium have shown that under basal conditions, there is a net secretion of Cl^-[94]. This model was chosen, as it demonstrates some similarities to mammalian alveolar epithelium[94]. However, it should be noted that in this study, there was evidence to support the presence a Cl^- channel, in addition to CFTR, which may contribute to the net secretion[94]. Additional evidence to support the observation that Cl^- secretion occurs in the lower airways is discussed by Brochiero *et al*., although it is stated that the exact site of secretion is not clear at this point[22]. Other studies performed using ATII cells (grown in an air-liquid interface) have indicated that the CFTR gene is present in the apical membrane[22,37]. Under basal conditions, it appears that fluid absorption is mostly driven through ENaC[37,98]. Highly active Na^+ absorption will depolarize the cell, favoring absorption of Cl^- through CFTR, upon stimulation with cAMP[37]. This absorptive characteristic appears to be dependent on the relative amounts of active ENaC present in the apical membrane[37]. Interestingly, it has also been shown that ATII cells grown in an air-liquid interface express higher amounts of active ENaC than those grown on submerged supports[38]. In general, an increase in active ENaC creates a drive for Cl^- absorption across the apical membrane in order to satisfy conditions of electroneutrality[37,38]. Additionally,

the model proposed by Johnson *et al.* indicates that ATI cells, in particular, contain multiple sodium channels (both selective and non-selective), further suggesting an absorptive phenotype in the lower airways[30,32] There is more evidence supporting the lower respiratory tract as having an absorptive phenotype than a secretory phentoype suggesting that the general movement of chloride on the apical membrane is inward[31,32,36].

Studies using mouse tracheal epithelium show that during the initial stages of influenza infection there is no impact on chloride conductance[60]. However, according to Wolk *et al*., after 2 days of infection with influenza virus, there was an observed increase in Cl^- secretion[61]. Hence, active viral replication is required in order to observe an effect on chloride conductance. The mechanism proposed by Wolk *et al.* involves the conversion of ATP into adenosine, which subsequently activates its receptor (A_1-subtype adenosine receptor) and stimulates the secretion of Cl^- through CFTR[61]. Moreover, it was also mentioned that UTP acts to increase Cl^- secretion, likely mediated through TMEM16A, in addition to decreasing Na^+ absorption[61,81]. The combination of these actions would result in increased fluid in the lungs.

Undoubtedly, Cl^- transport is heavily involved in the regulation of lung fluid homeostasis, and it seems that understanding the complex interactions that occur between cells in order to up- or down-regulate ion channels will remain an area of interest. These types of studies remain important, given that respiratory-associated infections and diseases afflict a large number of individuals each year. The above information demonstrates that influenza virus works on two levels to directly

modulate ion transport processes in respiratory epithelial cells to cause pulmonary edema. Initially, Na^+ absorption is decreased by binding of the virus to its cellular receptor; secondary to that, Cl^- conductance is modified by later stages of infection that involve viral replication (Figure 5)[60,61,62].

1.2.3. Potassium conductance

Although potassium conductance is essential for driving the movement of fluid in the airways, the impact influenza infection on this important process has not been studied. The movement of potassium (K^+) out of epithelial cells hyperpolarizes the membrane, creating a drive for the apical secretion of chloride[65,87,88,89,99]. In the upper airways, fluid secretion depends heavily on the ability to drive chloride across the apical membrane[11,12]. The main driving force for this movement of chloride is dependent on potassium channel activity, previously believed to exist only basolaterally[100,101]. However, more recent studies have indicated that both apical and basolateral K^+ channels are present, and play a role in regulating fluid secretion[88]. In Calu3 cells, the presence of the basolateral K^+ channel, KCNN4, has been shown, along with several apical K^+ channels (KCNQ family and K2P)[65,87,89,100].

KCNN4 is a calcium-activated potassium channel, which is primarily localized to the basolateral membrane of airway epithelial cells[65]. Increases in intracellular calcium stimulate the efflux of K^+ from this channel. As proposed by McCann *et al*., stimulation of tracheal cells with cAMP, in addition to increasing anion secretion through CFTR directly, will also increase cytosolic calcium concentrations[102]. This

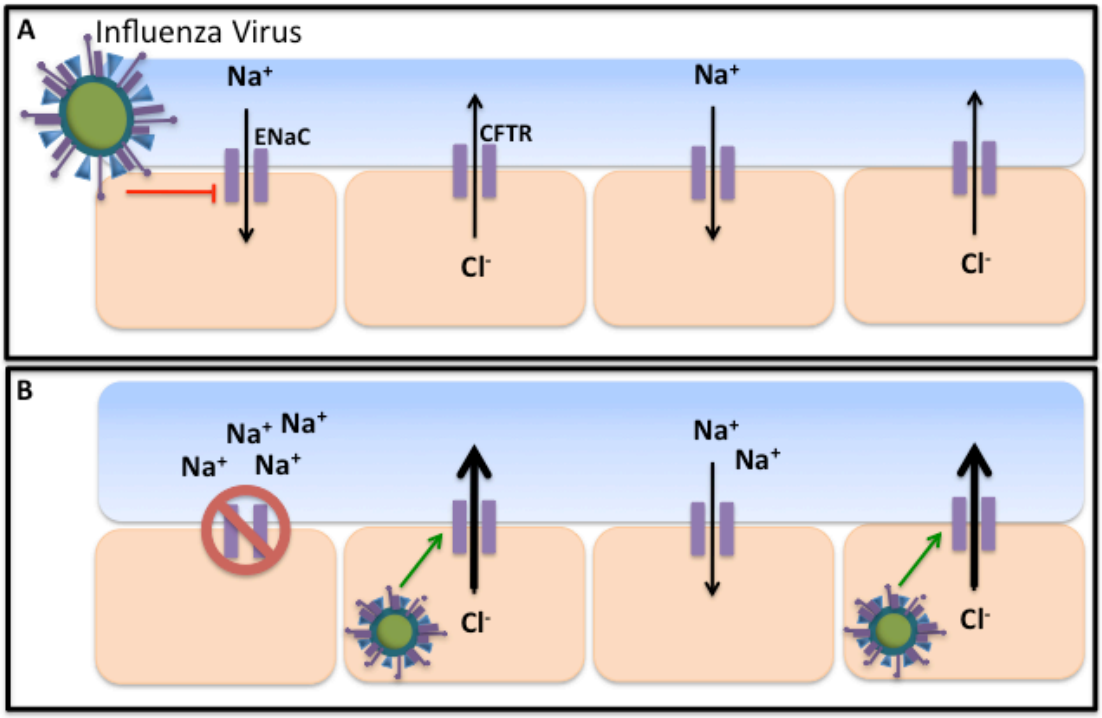

Figure 5. Direct impact of influenza virus infection on respiratory epithelial cell ion transport.

Simplified diagram showing the direct impact influenza virus infection has on ion transport processes. A) Short-term infection (after 1 hour) with influenza virus results in modulation of ENaCs via PKC activation (see Figures 3, 4). This initial binding of the virus to its cellular receptor leads to decreased ENaC activity (see text for further details). B) Longer-term infection involves active viral replication, which has been shown to stimulate secretion of Cl^- across the apical membrane (see text). The combination of these events prevents H_2O from being reabsorbed, leading to a buildup of fluid in the airspace (i.e., pulmonary edema).

will, in turn, activate basolateral potassium conductance and further drive apical anion secretion[100,102].

This model of recycling of potassium across the basolateral membrane (entry via Na^+-K^+-ATPase pump, exit through KCNN4) is based upon work done by Silva *et al*. in shark rectal glands[103]. However, more recent evidence suggests a role for apically located K^+ channels in driving anion secretion[87,88,89,91,104]. Cook *et al*. describes apical anion secretion being enhanced by the presence of active, apical K^+ channels[104]. Further work has been done in Calu3 and NHBE cells on the KCNQ family of cAMP-activated K^+ channels, as well as the two-pore K^+ channels (K2P), both of which are shown the be located in the apical membrane[87,88,89,91].

The KCNQ family of K^+ channels includes KCNQ1-5, which are cAMP-activated[89]. KCNQ1, in particular, is comprised of two subunits: KCNE2 and KCNE3[89,91]. Studies done in Calu3 cells indicate that KCNQ1 is present in both the apical and basolateral membrane, and contributes to the overall drive for apical anion secretion[65,89]. Cowley *et al*. describe KCNQ1 as a potential contributor, along with KCNN4, to the overall anion secretion in Calu3 cells; however, they failed to detect mRNA expression of both KCNQ1 subunits[65]. Moser *et al*. proposes that the ability of KCNQ1 to generate a cAMP-stimulated current is highly dependent on its association with both KCNE2/3[89]. Additionally, Moser *et al*. describes a larger role for other KCNQ family channels, namely KCNQ3 and KCNQ5, to the apical K^+ conductance observed in Calu3 cells[89]. It appears that Calu3 cells contain several different KCNQ channels, and that their expression levels may vary between cultures[89]. Additionally, studies in both Calu3 and NHBE cells indicate the

presence of a two-pore K^+ channel (K2P) located in the apical membrane[87,88]. The primary role of these K2P channels, and KCNQ1, is in maintaining hyperpolarizing conditions at the apical membrane in order to sustain chloride secretion[88]. The constant efflux of potassium, either through apical or basolateral channels, maintains the necessary negative membrane potential to drive secretion in the upper airways.

Influenza virus infection has been studied with regards to sodium and chloride ion transport; however, the effects on potassium conductance have yet to be addressed[60,61,62]. Clearly, the role of potassium conductance in the overall process of fluid secretion cannot be ignored, as they are inextricably linked. Research in this area may uncover potentially new drug targets for influenza-associated complications, like pulmonary edema.

1.3. Indirect effects of influenza on epithelial cell ion channel physiology

1.3.1. Activation of the innate immune system

The innate immune system acts as the first line of defense against any invading pathogens[13]. In the lungs, as previously mentioned, the ability to effectively clear the ASL and any trapped pathogens aid in defending against infections. This system is the primary defense mechanism; however, both epithelial cells and macrophages have the ability to further protect against invading pathogens through the production of cytokines[10,19]. Resident macrophages of the lungs are of critical importance in alerting the immune system during viral infections[46]. Macrophages are capable of phagocytosing invading pathogens,

which, in turn, activate them[13]. Upon activation, macrophages secrete several different pro-inflammatory cytokines, including TNF-α[46,47,105]. TNF-α is an important immune mediator, which is also produced by epithelial cells, and acts to recruit additional immune cells to the site of infection[47,48]. Additionally, TNF-α induces the secretion of other pro-inflammatory cytokines such as IL-6, IL-8 and IL-1α/β[48,106]. Together, these cytokines act to enhance the inflammatory response in the host[5,49]. However, certain cytokines have been shown to modulate ion transport processes, exacerbating the overall effect influenza virus has on respiratory epithelium[48,58,107,108,109].

Macrophages are not the only cells involved in stimulation of the innate immune response. In fact, the respiratory epithelial cells themselves are equipped with the ability to recognize PAMPs through a variety of different PRRs[53,54]. The most common PRRs involved in viral recognition include TLRs and RLRs (Figure 6)[52,53]. More specifically, TLR3 and TLR7 (located inside endosomes) have been implicated in recognition of influenza virus, as these receptors primarily recognize double-stranded and single-stranded RNA, respectively[49,53,55]. Furthermore, recognition of viral RNA in the cytoplasm is achieved through RIG-I[49,53,54]. The influenza virus RNA contains an uncapped 5'-triphosphate, and RIG-I has been shown to directly interact with this type of RNA[110]. Regardless of how influenza virus is recognized in the cell, there is initiation of signaling pathways which result in production of type I interferons (IFNs), as well as activation of NF-kB[54,55].

Type I IFNs include IFN-α and IFN-β; the expression of type I IFNs is critical in inducing an antiviral state inside the cell[49]. Induction of type I IFNs also aids in

the recruitment of various immune mediators such as dendritic cells and natural killer cells, which act to enhance the immune response[53,111]. Interestingly, influenza virus has some capacity to defend against type I IFN production. A study done by Kochs *et al*. has indicated that the non-structural protein, NS1, is able to antagonize the production of type I IFNs[112,113]. Further actions taken by the host innate immune response include activation of NF-kB. The NF-kB pathway is responsible for inducing the production of pro-inflammatory cytokines[52,53]. Overall, the recognition of viral PAMPs initiates fundamental pathways involved in limiting the spread of the virus. Interruptions to this process, mainly though interference by the virus itself can result in a reduced ability to clear the infection.

1.3.1.1. Role of pro-inflammatory cytokines

An important component of the innate immune response is mediated through the production of pro-inflammatory cytokines, namely TNF-α, IFN-α/β, IL-6, IL-8, RANTES and IL-1β[13]. There is evidence to support that infection of airway epithelial cells with influenza virus results in a strong pro-inflammatory cytokine response[5,106,114]. These cytokines, although important in limiting the spread of the virus, can also contribute to the overall pathogenicity of the infection[13,56,115]. Work done by Matsukura *et al.,* has indicated that human bronchial epithelial cells infected with influenza virus show increased expression of IL-6, IL-8 and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted)[56]. They further state that these cytokines may be contributing to the overall pathogenesis of influenza-induced airway inflammation[56,115].

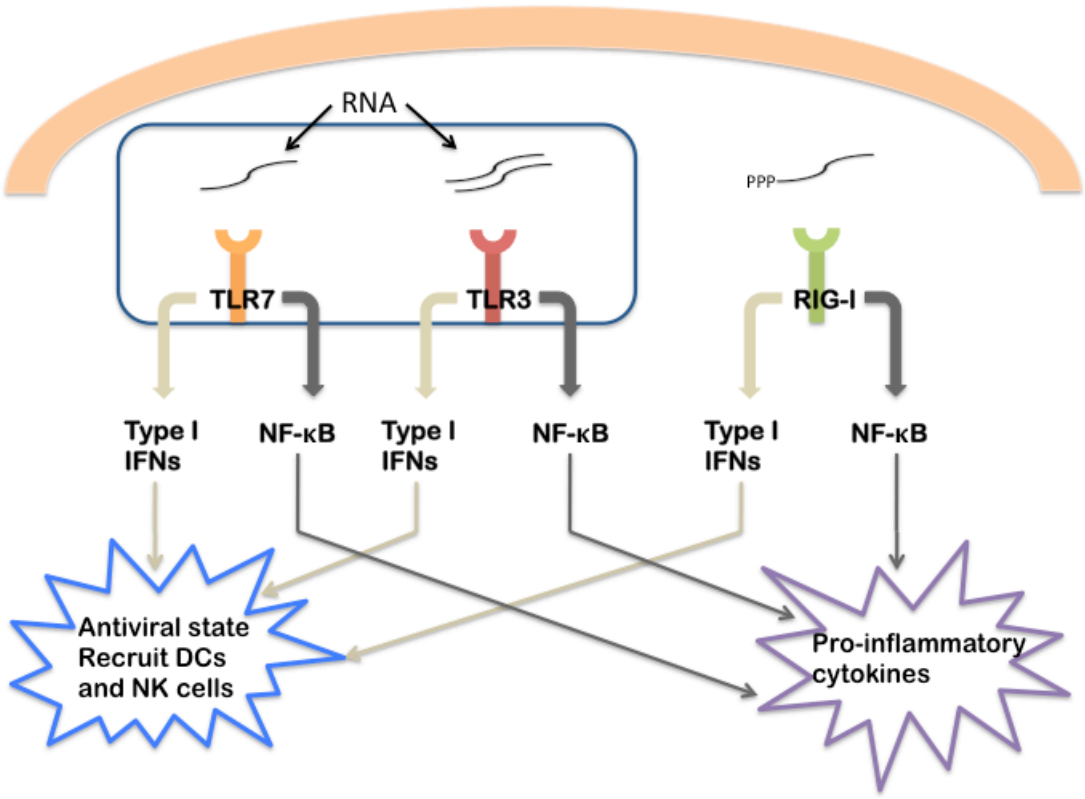

Figure 6. Outline of how epithelial cells recognize viral PAMPs.

Upon recognition of viral nucleic acid (dsRNA/ssRNA) either in the cytoplasm or inside endosomes, the innate immune system becomes activated. TLR3 and TLR7 are present inside endosomes, and recognize viral dsRNA and ssRNA, respectively. In the cytoplasm, uncapped 5'-triphosphate ssRNA is recognized by RIG-I. These pattern recognition receptors (PRRs) use different signaling pathways in order to induce an antiviral state, and recruit dendritic cells and natural killer cells through activation of type I interferons (IFNs). Pro-inflammatory cytokines are produced through the NF-kB pathway. Together, these components of the innate immune system aid in fighting viral infection.

In addition to this, studies have indicated that production of TNF-α and IL-1β are also increased during infection with influenza, and are found in edema fluid[48,116,117]. These inflammatory mediators have been implicated in regulating the ion transport processes in the airways[48,59,117,118].

Interleukin 1β (IL-1β) is considered to be one of the most biologically relevant inflammatory cytokines, especially in the context of edema formation in the lung[117]. Induction of IL-1β is associated with airway inflammation, and can be produced by a number of different cell types, including epithelial cells[59,119]. Moreover, IL-1β has been implicated as an inflammatory mediator that may directly impact ion transport processes in airway epithelial cells[59,117]. A study performed using human bronchial epithelium shows that after 48 hours of IL-1β treatment, there is a significant increase in basal currents, indicating there is a concerted effect on Na^+ absorption and CFTR-dependent Cl^- secretion[59]. This was further substantiated through direct measurement of ASL levels, which were increased after treatment with IL-1β[59]. Gray *et al*. has shown that long-term exposure to IL-1β results in a reduced ability to actively clear fluid from the airways, therefore contributing to the formation of lung edema. Complementary to this phenomenon, a study done using alveolar epithelial cells shows that IL-1β causes a decrease in αENaC mRNA and protein levels in a dose-dependent manner[117]. This effect was observed as early as 6 hours after treatment, and was ameliorated by pre-treatment with a receptor antagonist against IL-1β[117]. Upon further investigation into the specific mechanism, it was discovered that down-regulation of αENaC protein is mediated through the p38 MAPK signaling pathway[117]. This evidence supports

IL-1β as a key modulator of Na^+ transport during airway inflammation.

Several studies have already addressed the role TNF-α plays in modulating Na^+ transport processes in respiratory epithelium[48,118,120]. However, the exact role that this inflammatory cytokine plays in relation to Na^+ absorption is still under debate. A study that looked at Cl^- transport have shown that TNF-α is capable of modulating CFTR gene expression in a time and dose-dependent manner[120]. However, this study was performed using colon-derived epithelial cells, and therefore cannot be directly compared to respiratory epithelium. Work done by Fukuda *et al*. in alveolar epithelium has shown that immediately after the addition of TNF-α there is an observed increase in Na^+ transport[118]. They rationalize that this increased ability to transport Na^+ will lead to up-regulation of fluid clearance in the lung via ENaC[118]. Contrary to this, Dagenais *et al*. has shown that over a 24-hour period exposure to TNF-α results in a significant decrease in ENaC mRNA expression (α, β, and γ subunits) relative to untreated controls[48]. A decrease in apical ENaCs will lead to a decreased ability to clear fluid from the airways. These two studies imply very different outcomes for individuals suffering from influenza infection where expression of TNF-α has been shown to be increased. It seems likely that the data from the latter study are a better representation of what occurs in a natural infection, given that cytokines, like TNF-α, will be expressed for longer than the short time period assessed by the former study. Assuming that TNF-α does exacerbate edema formation during influenza virus infection, further investigation of the underlying mechanism may prove useful in the development of new drug therapies. Since TNF-α in particular is an important component of the innate

immune response, a targeted drug therapy may have universal applications that would extend beyond influenza virus infections.

Other cytokines that have been implicated in the modulation of both ENaC and CFTR in bronchial epithelial cells include IL-13, IL-4 and IFN-γ[107,108,109]. IL-13 and IL-4 are both Th2 cytokines, secreted in response to inflammation[108,109]. Cells dosed for 48 hours with IL-13 or IL-4 decreased sodium absorption, and increased both cAMP- and calcium-activated chloride secretion[109]. Further studies done by Galietta *et al*., indicate that IL-4 treatment down-regulates γENaC mRNA expression, accounting for the decreased sodium absorption[108]. Additionally, the observed increase in cAMP-dependent current, mediated by IL-4, was shown to be a result of increased CFTR mRNA expression[108]. IL-4 was capable of eliciting an increase in the calcium-dependent current after only 6 hours of treatment; however, the underlying mechanism that drives this up-regulation was not addressed[108]. IL-13 acts on the same epithelial receptor as IL-4, so although the details of how IL-13 works to induce hypersecretion in the airways were not directly addressed, it is likely that they work through a similar mechanism[108,109].

Interestingly, treatment of human bronchial epithelial cells with IFN-γ, which is produced in response to viral infection and contributes to the overall inflammatory response, resulted in down-regulation of cAMP-dependent chloride transport, but up-regulation of calcium-dependent chloride transport[107]. Galietta *et al*. attribute the reduction in cAMP-dependent transport to a down-regulation of CFTR mRNA, and suggest that IFN-γ may be acting directly on CaCCs to up-regulate

their activity[107]. Treatment with IFN-γ for 48 hours also reduced sodium absorption; however, there were no changes in ENaC mRNA[107,108]. Speculation in a follow up study indicates that ENaC is likely modulated through an alternative mechanism that has yet to be directly linked to IFN-γ[108].

The evidence presented here indicates that influenza virus is capable of indirectly modulating both Na^+ and Cl^- transport through activation of the innate immune system (Figure 7). Production of various inflammatory mediators, such as TNF-α, IL-1β, and IFN-γ, play a significant role in fighting against viral infection, and the initiation of the inflammatory response leads to anti-inflammatory cytokine production (i.e., IL-13, IL-4). However, these cytokines have the potential to exacerbate the damage caused by the infection through further modulation of ion transport processes. In addition to this, the pro-inflammatory cytokines recruit immune cells to the site of infection; infiltration of these cells causes inflammation, which, in the long term, will compromise the epithelial cell barrier[115]. Together, increased cellular permeability in conjunction with disrupted ion transport results in severe edema formation, and damage to the lungs.

1.4. Lung pathology

1.4.1. Epithelial cell damage

Given that influenza virus infections often lead to complications such as pulmonary edema, and pneumonia (discussed above), it is not surprising that the lungs themselves suffer extensive damage.

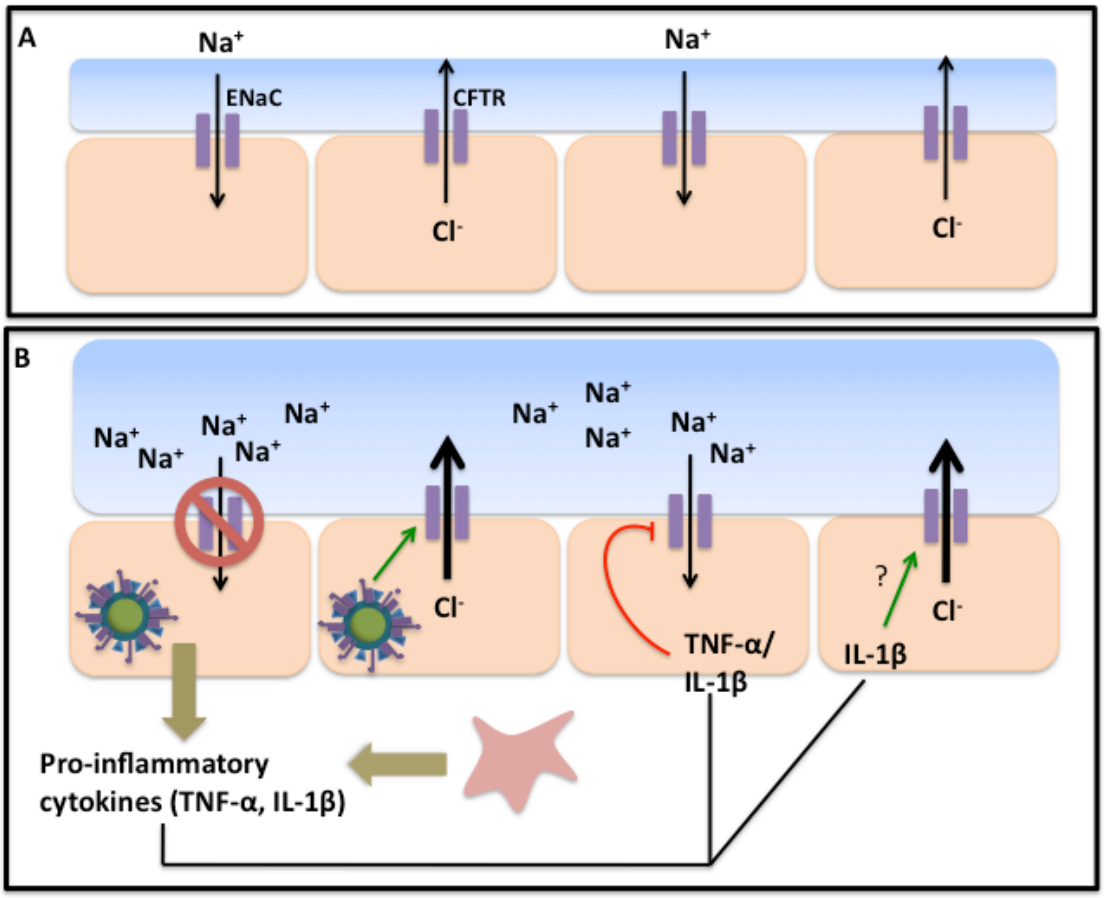

Figure 7. Overview of the indirect impact of influenza virus infection on ion transport processes.

Pro-inflammatory cytokines produced by epithelial cells infected with influenza virus can act on neighboring cells to modulate ion transport. A) Epithelial ion transport at homeostasis (simplified from figure 1). Initially, virus infection alters ENaC, reducing the ability to reabsorb Na^+ (see text, and figures 3, 4). There is no observed effect on chloride secretion upon initial infection. B) Longer-term impact. After viral replication begins, there is an up-regulation of apical Cl^- secretion. Epithelial cells begin secreting pro-inflammatory cytokines and evoke the innate immune response (involves activation of macrophages, which contribute to the pro-inflammatory response). Although many different cytokines, both pro- and anti-inflammatory, are produced, TNF-α, IL-1β, and IL-4 have the capacity to further down-regulate ENaC (see text for details). IL-1β and IL-4 may also be involved in up-regulating CFTR, whereas IFN-γ has been shown to modulate calcium-activated chloride channels. Altogether, this leads to a reduced ability to clear fluid from the airspace, resulting in pulmonary edema. Recruitment of additional immune cells to the site of infection leads to further inflammation, which contributes to cellular damage in the lungs.

The damage caused by infection is a result of both the cytopathic effect of the virus, combined with the inflammatory response of the host[7]. The NS1 viral protein has been implicated as a major contributing factor to the overall pathogenesis of influenza[113,121]. One study identified that NS1 is likely to be involved in the induction of pro-inflammatory cytokines in addition to stimulating strong apoptotic responses[121]. The induction of apoptosis, along with a strong inflammatory response has major implications for disease severity[105,121]. In general, fatal cases of influenza virus infection require movement of the virus into the lower respiratory tract[3,7]. Those individuals infected only in the upper respiratory tract develop clinical symptoms such as bronchitis, whereas more severe cases with lower respiratory tract involvement can result in diffuse alveolar damage, which can lead to fatal respiratory dysfunction[3,7,35].

In acute stages of infection, there is a large degree of variability in structural damage to the epithelium. A common theme, however, is desquamation of epithelial cells, as well as apoptosis and necrosis[3,105]. As the infection progresses, both macrophages and neutrophils begin to infiltrate the airspace; these cells will phagocytose the apoptotic and necrotic epithelial cells[7,105]. Furthermore, the development of viral pneumonia is associated with alveolar hemorrhaging and necrotizing bronchiolitis[3,114]. Additional complications include secondary bacterial infections, which aggravate the inflammatory response, leading to further infiltration of neutrophils[3].

The majority of studies looking at lung pathology occur in individuals who have succumbed to complications, making it difficult to ascertain the damage caused

in the initial stages of the infection. It is important to note that the severity of influenza virus infections arises from a multitude of factors, both virus- and host-related. Additionally, not all of the cells in the airway will become infected with the virus, making the associated pathology a result of both virus and host factors.

1.5. Conclusion

Influenza virus uses a multifaceted approach to cause disease in the airways. The virus acts directly on epithelial cells to alter the transport of Na^+ and Cl^-, at the ion channel level. As the infection progresses and viral replication ensues, a range of signaling pathways contribute to further disruption of ion transport. In general, there is an observed decrease in the ability to reabsorb Na^+, along with an increase in Cl^- secretion. Potassium channels, which create the drive for chloride secretion, are likely involved; however, this area has yet to be studied. These processes impair the natural capacity of the lungs to reabsorb fluid from the airspace, resulting in pulmonary edema. The immune response of the host also contributes to this phenomenon, further exacerbating the situation. The combined effects of the virus and the host immune response often lead to fatal complications due to extensive lung damage. Further investigations into the complex mechanisms that underlie the pathophysiology are required to eliminate influenza-associated fatalities.

1.6. Purpose

Evidence indicates that influenza virus is capable of directly modulating both sodium and chloride ion channels in epithelial cells. Additionally, the pro-inflammatory cytokines produced by the infected epithelial cells have been shown

to have regulatory effects on these same ion channels. However, the effects of influenza virus infection have not yet been studied with respect to potassium ion channels. This is important because the movement of potassium out of the cell directly influences the movement of chloride and this process regulates airway hydration. The purpose of this study is to assess both the direct and indirect effects of influenza virus infection on potassium and chloride channel function in the secretory cell model, Calu3.

1.6.1 Hypothesis

The majority of the epithelium remains uninfected after initial exposure to influenza virus. The cells that have been infected will secrete both pro- and anti-inflammatory cytokines, which can act in a paracrine manner on the non-infected cells. There is evidence showing that certain cytokines are capable of modulating ion channel function in respiratory epithelium. Therefore our hypothesis is that the inflammatory cytokine mixture secreted by epithelial cells during influenza virus infection is capable of modulating chloride and/or potassium ion channel function independently of the virus. In order to test this hypothesis, the direct and indirect effects of influenza virus infection were studied separately to determine whether the virus was required to modulate chloride and potassium ion channel function.

1.6.1.1. Specific objective 1

To determine if exposure of Calu3 cells to influenza virus for 24 and 48 hours at a high dose (multiplicity of infection (MOI) of 10) will modulate chloride and/or

potassium ion channel function. We hypothesized that a high viral dose will decrease chloride and potassium ion channel conductance.

1.6.1.2. Specific objective 2

To determine a physiologically relevant dose (lower dose) of influenza virus that will induce an immune response and modulate ion channel physiology so as to model the symptoms observed in a natural infection, i.e., pulmonary edema. We hypothesized that since not all of the cells in the respiratory tract are infected during a natural infection, a multiplicity of infection between 0.1 and 1 should be sufficient to model the effect of both virus, and cytokines together *in vitro*.

1.6.1.3. Specific objective 3

To determine whether the cytokines produced after 48 hours of infection are capable of independently modulating chloride and/or potassium ion channel function. We hypothesized that the mixture of cytokines produced during infection with influenza will cause an increase in chloride and/or potassium ion channel conductance.

1.6.1.4. Specific objective 4

To determine whether direct exposure to a single pro-inflammatory cytokine, interleukin-8 (IL-8) for 48 hours, will induce similar changes in chloride and potassium ion channel conductance as the cytokine mixture. Our hypothesis was that exposure to IL-8 for 48 hours will result in an increase in chloride and/or potassium ion channel function.

CHAPTER 2. MATERIALS AND METHODS

2.1. Cell culture

The human bronchial epithelial cell line, Calu3 (HTB-55; ATCC), was cultured at a low passage number in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (16000-044; Life Technologies), 1% Non-essential amino acids (10 mM, Invitrogen), 1% Penicillin-Streptomycin (Invitrogen), 1% Sodium Pyruvate (Invitrogen), and 1% L-glutamine (200 mM, Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2 [65]. Cells were then seeded onto 0.4-μm pore size Snapwells (Corning) and grown until they formed a confluent monolayer, as previously described[65,89]. Once confluent, the apical media was removed and cells continued to grow in an air-liquid interface for approximately one week prior to treatment.

2.2. Viral culture and infection

Polarized Calu3 cells were infected apically with A/PR/8/34 H1N1 at varying multiplicity of infection (MOI). Influenza A virus was propagated at a MOI of 0.001 in Madin-Darby canine kidney (MDCK) cells and purified by ultracentrifugation through a sucrose gradient. Stock virus concentration was determined using a plaque assay (see section 2.3.1.) and quantified in terms of plaque-forming units (PFU) per cell. The virus was diluted in DMEM and 0.5 mL of the inoculum was added to the apical surface of the cells at a MOI of 10, 1, 0.6, or 0.3. Cells were infected apically for 1 hour, after which they were washed with phosphate buffered saline (PBS; 137mM NaCl, 2.6mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4) to remove

non-adherent virus, and fresh DMEM was replaced on the apical surface. Similarly, the control cells were mock infected using DMEM only. Treated cells were left for 24 or 48 hours before performing any downstream experiments.

2.3. Conditioned media

Four types of conditioned media were generated from non-polarized, confluent Calu3 monolayers in 6-well plates (Corning): FBS-free control conditioned media (FBS-free CCM), FBS-free virus-treated conditioned media (FBS-free VCM), control conditioned media with FBS (FBS+ CCM), and virus-treated conditioned media with FBS (FBS+ VCM). The VCM was generated using an MOI of 10. Cells were rinsed with PBS after one hour of infection, and 3 mL of fresh media (FBS-free or FBS+) was replaced into each well. After 48 hours, the media was transferred to a sterile 6-well plate 30 cm from an UV light for 30 minutes[122]. A plaque assay was used to confirm that the virus particles had been completely inactivated[123].

2.3.1. Plaque assay

Using 6-well plates, MDCK cells were seeded at 6 x 10^5 cells/well 16-18 hours prior to assay. MDCK cells were cultured in Modified Eagle's Medium (MEM, HyClone) supplemented with 10% FBS, 1% Penicillin-Streptomycin and 1% L-glutamine. Serial dilutions of the UV-treated conditioned media were prepared along with a positive and negative control. Media was removed from MDCK cells, and each well was rinsed with PBS. 400 μL of each sample dilution were added to the wells in duplicate and the plate was incubated at 37°C for 1 hour. The inoculum was then removed from each well, rinsed with PBS, and replaced with 3 mL of media

overlay. The media overlay contains 20 mL 2X MEM (supplemented with 2% penicillin-streptomycin, 2% L-glutamine), 1.6 mL 5% bovine serum albumin (BSA), 40 μL TPCK trypsin (1 mg/mL; 20233, Thermo Scientific), and 20 mL 1.8% agarose. The overlay was allowed to solidify for 10-15 minutes before being placed back in the incubator at 37°C. After 72 hours, the agarose-media overlay was carefully removed, and the cells were stained with 2 mL Coomassie blue (50% CH_4O, 10% $C_2H_4O_2$, 40% H_2O, 0.25% Coomassie Brilliant Blue R).

2.4. Quantification of cytokines

Cytokine levels in the conditioned media were quantified using the Bio-Plex Pro assay according to the manufacturer's instructions (Bio-Rad)[4]. Included in the assay was IL-4, IL-6, IL-8, IL-10, TNF-α and IFN-γ.

2.4.1. Cytokine dose response

Interleukin-8 (IL-8; Sigma; SRP3098) was reconstituted in water and used to dose polarized Calu3 cells for 48 hours. Cells were dosed apically (DMEM) and basolaterally (culture media) at either 10, 100, or 1000 ng/ml of IL-8[108]. The IL-8 containing media was changed after 24 hours and cells were left for a total of 48 hours. Downstream experiments involved measurement of transepithelial ion transport in an Ussing chamber, and subsequent RNA extraction for RT-qPCR.

2.5. Measurement of transepithelial short-circuit current (I_{sc})

Polarized cells on Snapwells were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA) (Figure 8). The apical and basolateral chambers were filled with 5 mL Kreb's solution containing (in mM): 113 NaCl, 5 KCl, 1.6 Na_2HPO_4,

0.3 $NaH_2PO_4 \bullet H_2O$, 25 $NaHCO_3$, 1.1 $MgCl_2 \bullet 6H_2O$, 2.2 $CaCl_2 \bullet 2H_2O$, and 10 glucose[124]. Chambers were maintained at 37°C and bubbled with 5% CO_2-95% O_2. Transepithelial potential differences were short-circuited to 0 mV with a voltage clamp on the apical and basolateral chambers using Ag-AgCl electrodes and 3M KCl agar bridges[108]. Cells were clamped, and allowed to equilibrate for a minimum of 20 minutes prior to any addition of drugs (see table 1). Each drug was added at 6-8 minute intervals. Tissue was pulsed every 30 seconds with a 1 mV pulse and the resulting current was used to determine membrane resistance. Positive current deflection is the result of either an anion moving apically, or a cation moving basolaterally.

2.5.1. Chemicals

All chemicals and drugs were obtained from Sigma, unless otherwise noted. Forskolin (10 μM; F6886) and 3-isobutyl-1-methylxanthine (IBMX, 1 mM; I5879). Calcium ionophore A23187 (500 nM; C7522). Glibenclamide (100 μM; G0639). Niflumic Acid (100 μM; N0630). TRAM-34 (20 μM; T6700), Chromanol 293B (30 μM; C2615), and ICA-17043 (100 μM; kindly provided by Dr. Heike Wulff from the University of California).

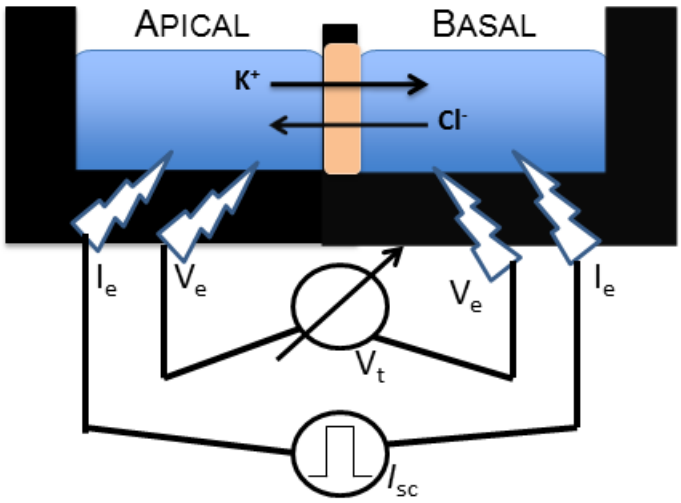

Figure 8. Ussing chamber model.

This tool allows for the measurement of short-circuit current (I_{sc}), which indicates the net ion transport occurring across a polarized epithelium. Cells mounted in the chamber separate the apical and basolateral sides, which are bathed in symmetrical solution (e.g., Kreb's solution), thereby eliminating any electrochemical gradient. Two voltage electrodes (V_e) measure the potential difference, which is cancelled out by the imposed current from the current electrodes (I_e). This current is referred to as short-circuit current, and represents the net movement of ions across the epithelium. The use of channel agonists and blockers will alter the membrane resistance by opening or closing ion channels, which results in measurable changes in I_{sc}.

Table 1. Drug names and actions

Drug Name	Channel Modulation	Channel Activator or Inhibitor
Forskolin/IBMX	CFTR	Activator
A23187	Ca^{2+}-activated channels	Activator
Glibenclamide	CFTR	Inhibitor
Niflumic Acid	Ca^{2+}-activated Cl^- channels	Inhibitor
TRAM-34	KCNN4	Inhibitor
ICA-17043	KCNN4	Inhibitor
Chromanol293B	KCNQ1	Inhibitor

2.6. RNA isolation and Real-Time Quantitative PCR (RT-qPCR)

RNA was extracted using TRIzol Reagent (15596018; Life Technologies) according to the manufacturers protocol. cDNA was transcribed using GoScript Reverse Transcription system (A5001; Promega). cDNA concentrations of approximately 100-150 ng were measured by real-time quantitative PCR using GoTaq qPCR Master Mix (A6002; Promega), with Stratagene MX5000P real-time PCR machines. See table 2 for list of primers.

2.6.1. Efficiency and fold difference calculation

Dilution series from 1×10^{0} fold – 1×10^{-6} fold of cDNA were used to determine the primer efficiency. The C_T value obtained in each dilution was used to generate a linear plot of C_T vs. log copies graph. The efficiency of the primer set is determined with the equation:

$$\text{Efficiency } (\%) = 10^{(-1/\text{slope})}$$

The fold difference between infected/treated and non-infected cells was determined using efficiency corrected calculation model:

$$\text{Ratio} = \frac{(\text{Eff}_{\text{target}})^{\Delta CT \text{ target } (\text{Mean control} - \text{Mean sample})}}{(\text{Eff}_{\text{ref}})^{\Delta CT \text{ ref } (\text{Mean control} - \text{Mean sample})}}$$

Table 2. Primer sequences used for RT-qPCR.

Gene Name	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Tm (°C)
GAPDH	CAA GGT CAT CCA TGA CAA CTT TG	GGG CCA TCC ACA GTC TTC TG	60
ACTB	ACC AAC TGG GAC GAC ATG GAG AAA	TAG CAC AGC CTG GAT AGC AAC GTA	60
CFTR	TCC TCC AAA CCT CAC AGC AAC TCA	AAG GCA CGA AGT GTC CAT AGT CCT	60
KCNN4	GGC CAA GCT TTA CAT GAA CAC GCA	AAA GGT GCC CAG TGG CAT TAA CAG	60
KCNQ1	TCC TCG TAC TTT GTG TAC CTG GCT	AAA GAC AGA GAA GCA GGA GGC GAT	60
NKCC1	CAT TAA CTG GTG GGC TGC ATT GCT	TCA CGT GGT CTT CCA CTC CAG AAA	60
ATP1A1	GGG CAA ATG TCG AAG TGC TGG AAT	TTC CAC GGT CTC ATT GCC TTC TGA	60
IL-6	AGC CAC TCA CCT CTT CAG AAC	GTG CCT CTT TGC TGC TTT CAC	57
IL-8	TCT CTT GGC AGC CTT CCT GAT TTC	ATT TCT GTG TTG GCG CAG TGT G	60
IL-12α	CAG TGG AGG CCT GTT TAC CAT TG	TAC TAC TAA GGC ACA GGG CCA TC	58
IL-1α	TAC CTC ACG GCT GCT GCA TTA C	GGT CTT CAT CTT GGG CAG TCA C	58
IL-1β	GCT GAT GGC CCT AAA CAG ATG	TGT AGT GGT GGT CGG AGA TTC	55
TNF-α	TGC TGC ACT TTG GAG TGA TCG	TGC TAC AAC ATG GGC TAC AGG	57
IFN-α	TCT CAG CAA GCC CAG AAG TAT C	GAG CAT CAA GGT CCT CCT GTT ATC	56

2.7. Western blot analysis

Protein was extracted from Calu3 cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (78833; Pierce) and analyzed the same day. Individual concentrations were determined using Bradford Assay (Bio-Rad)[125]. The samples were boiled in 2x denaturing buffer (20% glycerol, 4% SDS, 125mM Tris pH 6.8, 0.3mM bromophenol blue) and analyzed by 10% SDS-PAGE. After SDS-PAGE, proteins were electroblotted onto PVDF membrane (RPN303LFP; GE Healthcare Life Sciences) with transfer buffer (25mM Tris, 192mM glycine, 20% methanol)[94]. Membranes were blocked overnight at 4˚C with 5% bovine serum albumin in PBST buffer (137mM NaCl, 2.6mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 0.1% Tween-20; Sigma) and subsequently probed for 2h at room temperature with anti-CFTR (1:200; sc-10747; Santa Cruz)[94], anti-KCNN4 (1:200; sc-32949; Santa Cruz), anti-KCNQ1 (1:200; sc-20816; Santa Cruz), or anti-GAPDH (1:500; sc-25778; Santa Cruz)[89]. Infrared-labeled secondary antibodies were used to visualize protein. Densitometry analyses of individual bands were compared for statistical analysis.

2.8. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Change in I_{sc} between treated and control cells were analyzed using parametric t-tests, or ANOVA, as appropriate. One-sample t-tests were performed on fold differences from data obtained using RT-qPCR with the mean set as 1. Significance was determined at $p < 0.05$.

CHAPTER 3. INFLUENZA A (H1N1) INCREASES AIRWAY SECRETION THROUGH UP-REGULATED POTASSIUM CHANNEL KCNN4. A NEW TARGET FOR THE TREATMENT OF ACUTE FLU

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3.1. Abstract

Influenza A infection of respiratory epithelium lining the airways produces cytokines that aid in fighting the infection. These same cytokines by themselves have been shown to modulate ion channel function. Movement through these channels creates the osmotic drive to hydrate the airways. However, the excessive increase in cytokine production during an influenza infection could contribute to excessive chloride and potassium channel activity and airway over-hydration. This could explain, in part, the disruption of the fluid balance in the lungs and the resulting pulmonary edema that occurs during severe influenza infections. Using RT-qPCR we measured cytokine and ion channel expression in the Calu3 cell line after 48-hour infection with influenza A virus (A/PR/8/34 H1N1). We simultaneously measured chloride and potassium channel function by means of a short-circuit current (I_{sc}) produced by a Calu3 cell monolayer in an Ussing chamber. At an MOI of 10, viral M1 protein and pro-inflammatory cytokine expression was observed 24 hours post-infection, despite a lack of measurable change in I_{sc}. However, we observed a decreased secretory response in cAMP- and calcium-induced I_{sc} 48 hours post-infection. This was a result of a virus-induced decrease in CFTR and KCNN4 protein. Interestingly, a viral dose response revealed an increased secretory response at an MOI of 0.6 ($p = 0.015$). This response correlated strongly with increased pro-inflammatory cytokine expression, suggesting a paracrine regulation of non-infected cells. This secretory response seems to be primarily driven through KCNN4, as up-regulation of both mRNA ($p = 0.005$) and protein ($p < 0.001$) levels were detected while CFTR function and expression remained

unchanged. Furthermore, inhibition of the KCNN4-stimulated I_{sc} with TRAM-34, a specific inhibitor, ameliorated the response, implicating KCNN4 as the main driving force behind the secretory phenotype.

3.2. Introduction

Seasonal circulating strains of influenza A virus infect millions of individuals worldwide[8]. These infections result in over half-a-million deaths annually, with mortality rates substantially higher when novel pandemic strains emerge[126]. The deaths are primarily due to influenza's ability to infect and disrupt the basic cellular mechanisms of ciliated epithelial cells lining the upper respiratory tract[127,128,129]. These cells are normally responsible for regulating the airway surface liquid (ASL), a thin film of fluid that covers the airways[11,130]. The ASL, maintained at appropriate volumes, allows the cilia of the epithelium to beat, clearing the lungs of mucus and inhaled contaminants (referred to as mucociliary clearance)[10,18,19]. The cells maintain the ASL by establishing an osmotic gradient across the epithelium[11,39]. This process is driven by the transport of sodium, chloride and potassium through ion channels and pumps[11,12]. Simply, potassium exits through ion channels on the apical and basolateral sides of the polarized epithelium, increasing the negative charge within the cell[11,89,91]. This, in turn, drives chloride into the airways through ion channels on the apical side, generating the gradient for both sodium and water to follow[11,28]. Chloride exits the apical membrane either through the cAMP-activated cystic fibrosis transmembrane regulator (CFTR) channel, or calcium-activated chloride channels (CaCC)[11]. The importance of CFTR is illustrated by its mutational loss in the disease of cystic

fibrosis (CF). In CF, the lack of CFTR results in under-hydration of the airways leading to decreased ASL levels and impairment of mucociliary clearance [37,97,131]. Alternatively, increased movement of chloride through CFTR, or CaCC, results in over-hydration of the airways, which can lead to pulmonary edema[33,40]. If not corrected, this edema formation can ultimately drown the patient[33]. Such severe pulmonary edema is particularly salient in patients suffering from acute, complicated influenza infections[11,40,61].

The exact mechanism by which influenza A virus infection can cause the formation of pulmonary edema remains unclear [23]. Initial studies have identified that the interaction of the virus with the epithelium leads to a reduced ability to clear fluid from the airways [60,61,62,76]. These studies specifically emphasize how down-regulation of the epithelial sodium channel (ENaC) impacts fluid clearance, as a direct result of viral attachment [60,62,76]. Since ENaC is responsible for absorbing sodium from the airways, this down-regulation reduces the net movement of water from the apical side[12]. This can ultimately lead to the formation of pulmonary edema in acute influenza infections[60,61,76].

However, the formation of pulmonary edema could also result from increased secretion, rather than decreased absorption (fluid clearance). This may be true in a natural infection where a large percentage of the cells are not infected and, instead, subject to paracrine regulation by infected cells[132,133]. Upon infection, epithelial cells respond by releasing a variety of inflammatory mediators[50,129]. These mediators are cytokines that aid in fighting the infection, and some, such as TNF-α, IL-1β, and IFN-γ, have been shown to independently increase net anion

secretion[48,58,107]. This results in increased airway fluid volume and is driven primarily through cytokines acting on non-infected cells[48,58,134]. Such an effect would most likely be mediated through increased CFTR activity on the apical membrane or increased potassium movement through the major epithelial potassium channels KCNN4 or KCNQ1[11,89].

To investigate the presence of a secretory airway response to influenza A virus infection, we used the well-established secretory airway epithelial serous cell model, Calu3[63,86]. The underlying drive for chloride secretion in this cell line comes from a small apical CaCC, highly abundant CFTR, as well as the potassium channels KCNN4 and KCNQ1[24,63,65,86,135]. Using this cell line we determined the effect of an influenza virus dose response on chloride and potassium channel expression and function as measured by Ussing chamber I_{sc}. Initially, we sought to characterize the effects of influenza virus infection on polarized Calu3 cell monolayers and assess the innate immune response elicited by our cell model. Our goal was to model the *in vivo* situation where only a small percentage of the airway cells are infected, leaving the non-infected cells available for paracrine regulation by virus-induced mediators, such as pro-inflammatory cytokines.

3.3. Materials and Methods

Refer to chapter 2

3.4. Results and discussion

To determine the effect of influenza A infection and replication on transepithelial ion transport, as a measurement of epithelial secretion we infected

polarized Calu3 cell monolayers with influenza virus (H1N1) at an MOI of 10 (i.e., 10 virus particles per cell). The initial use of a high MOI (Figures 9 and 10) was used for several reasons. This ensured that all cells were infected, and allowed us to characterize the immune response. Influenza-related fatalities resulting from pulmonary edema are often associated with higher viral loads and a strong inflammatory response [133,136]. Additionally, this initial high viral dose circumvented any problems associated with pandemic H1N1 eliciting an attenuated immune response, as this phenomenon has been previously reported[4,66].

Unexpectedly, 24 hours after infection despite the beginnings of a strong immune response and viral replication, as determined by cytokine transcript accumulation (Figure 9E) and M1 viral protein production (Figure 9F,G), there was no statistical change in epithelial resistance, a measure of tight junction permeability. Infected cells had a resistance of 90.8 ± 8.34 Ωcm^2 whereas non-infected cells had a resistance of 105.3 ± 8.51 Ωcm^2. Additionally, we observed no difference in cAMP- or calcium-induced I_{sc}, a measurement of epithelial secretion, between infected and non-infected epithelium (Figures 9A-D). We had expected to detect a change in I_{sc} after 24 hours of infection at an MOI of 10, as previous studies had found an impact on sodium absorption within a few hours[60]. These studies only assessed the direct impact of influenza virus during the early stage processes of viral attachment, and endocytosis, occurring within a 1-hour period[60,61]. These early time point events do not take into account the dynamic virus-host interactions that occur upon initiation of viral replication, as the average incubation period for influenza is 24-48 hours[8].

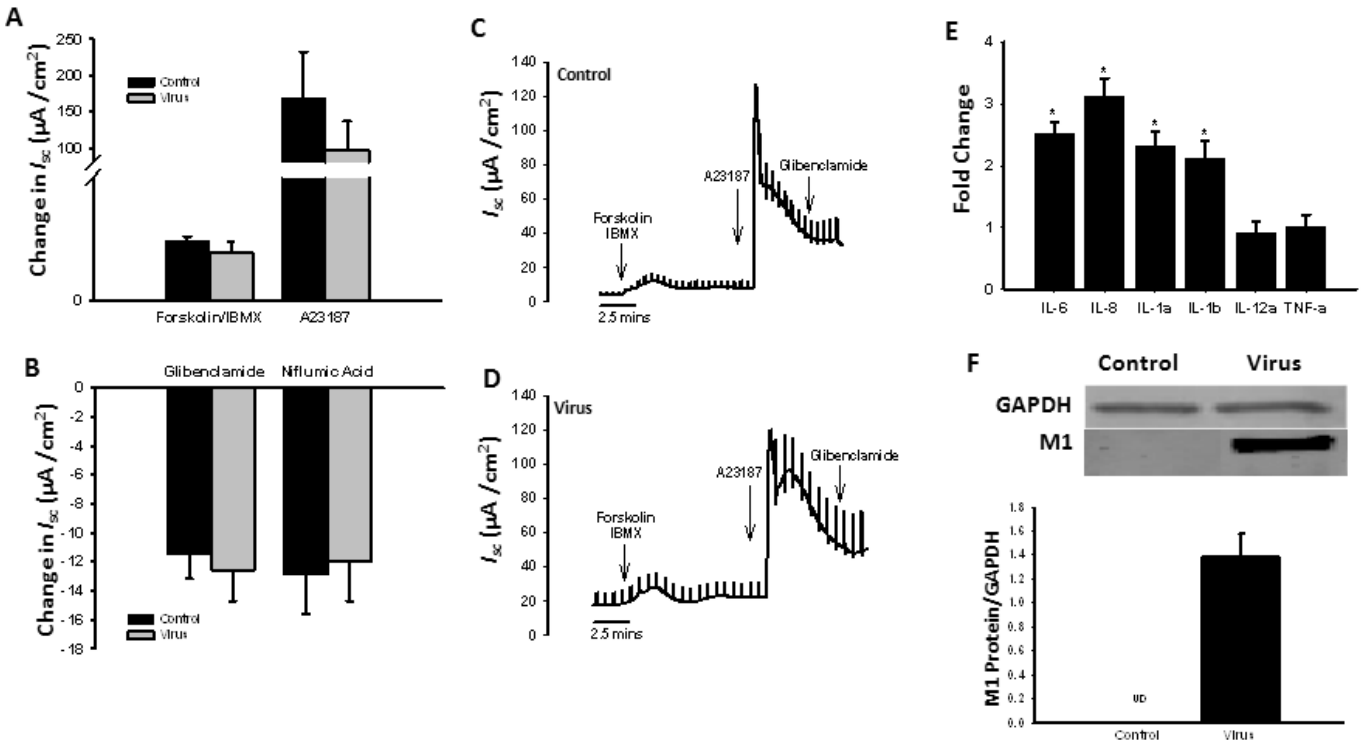

Figure 9. Effect of a high viral dose of influenza (MOI 10) on Calu3 cells 24 hours post-infection.

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A) or inhibitors (glibenclamide and niflumic acid; B) in control and virus-infected cells. Experimental I_{sc} traces representing cellular responses to activating and inhibiting drugs among control (C) and virus-infected (D) cells. Change in cytokine mRNA, as measured by RT-qPCR (E). Western blot and densitometry analysis of influenza virus M1 protein (~ 31 kDa) compared to GAPDH (~37 kDa) reference (F). I_{sc} = short-circuit current; UD = undetectable; Data presented as mean ± SEM, and analyzed using t-test. * = $p < 0.05$

Furthermore, viral shedding and the induction of apoptosis occur as late as 4 days post-infection[8]. Thus, we had predicted that 24 hours post-infection would reveal a difference in ion channel physiology, in addition to increased cytokine levels. However, we only detected significant increases in mRNA of IL-6, IL-8, IL-1α and IL-1β (Figure 9E) and no change ion channel function. We concluded since all cells were infected that, in our cell model, more time was required in order to observe a measurable difference in ion channel transport. Therefore, we proceeded to assess the impact of virus infection on cells at 48 hours post-infection.

Interestingly, after 48 hours at an MOI of 10 we observed a decreased secretory response, as indicated from a significant decrease in the cAMP- and calcium-activated I_{sc} of the infected cells (Figure 10A). Also, there was decreased chloride conductance after inhibition with glibenclamide, but not niflumic acid (Figure 10B). Moreover, the epithelial resistance of infected cells was significantly decreased compared to controls (61.5 ± 7.02 Ωcm^2 and 145.1 ± 21.2 Ωcm^2, respectively; $p = 0.002$). It is known that prolonged exposure to influenza virus leads to apoptosis [23,35,133]; therefore, the significant decrease in epithelial resistance, especially at this high dose, was not surprising. Despite detecting a decreased secretory response the reduction in epithelial resistance would compromise the epithelial barrier. The weakening of the epithelium would lead to the development of pulmonary edema because hydrostatic pressure would force fluid into the airway [39]. Chen *et al.* discuss that part of the pathogenesis of influenza is a result of an increased viral load damaging the epithelial barrier such that the patient is unable to recover from the massive flooding of fluid [62]. This

exacerbates the direct effects influenza has on ion transport in a functioning epithelium, which has been shown to also lead to pulmonary edema[60].

Interestingly, this decreased secretory response was not due to a decrease in transcription of the transporters responsible for driving secretion. RT-qPCR analysis of mRNA levels for CFTR, KCNN4, or the basolateral Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) and Na^+-K^+-ATPase (ATP1A1) were unchanged (Figure 10E). Further investigation using western blotting indicates that both CFTR and KCNN4 protein levels are significantly decreased in the infected cells ($p = 0.002$; $p = 0.022$; Figure 10F, G). Given that mRNA levels remained unchanged, it appears that at a high viral dose, there is post-transcriptional modification of both the CFTR and KCNN4 ion channels, resulting in an overall decrease in transepithelial I_{sc}. A possible explanation for this phenomenon is during influenza virus replication the viral polymerase subunit, PA, utilizes a cap-snatching mechanism in order to transcribe the viral genome—a process involving removal of host 5' mRNA caps[137]. These uncapped host mRNAs are then targeted for degradation, as they cannot be translated without a 5' cap. Despite the fact that there have not been any reports regarding the specificity of the viral polymerase to any host genes, the process may partially explain the reduced ion channel protein expression. Since we detected no change in ion channel mRNA, it is possible that the mRNA expression in the infected cells is being up-regulated, but with the intense viral replication any up-regulation of mRNA may be masked, which is why we only detected significant changes in protein expression.

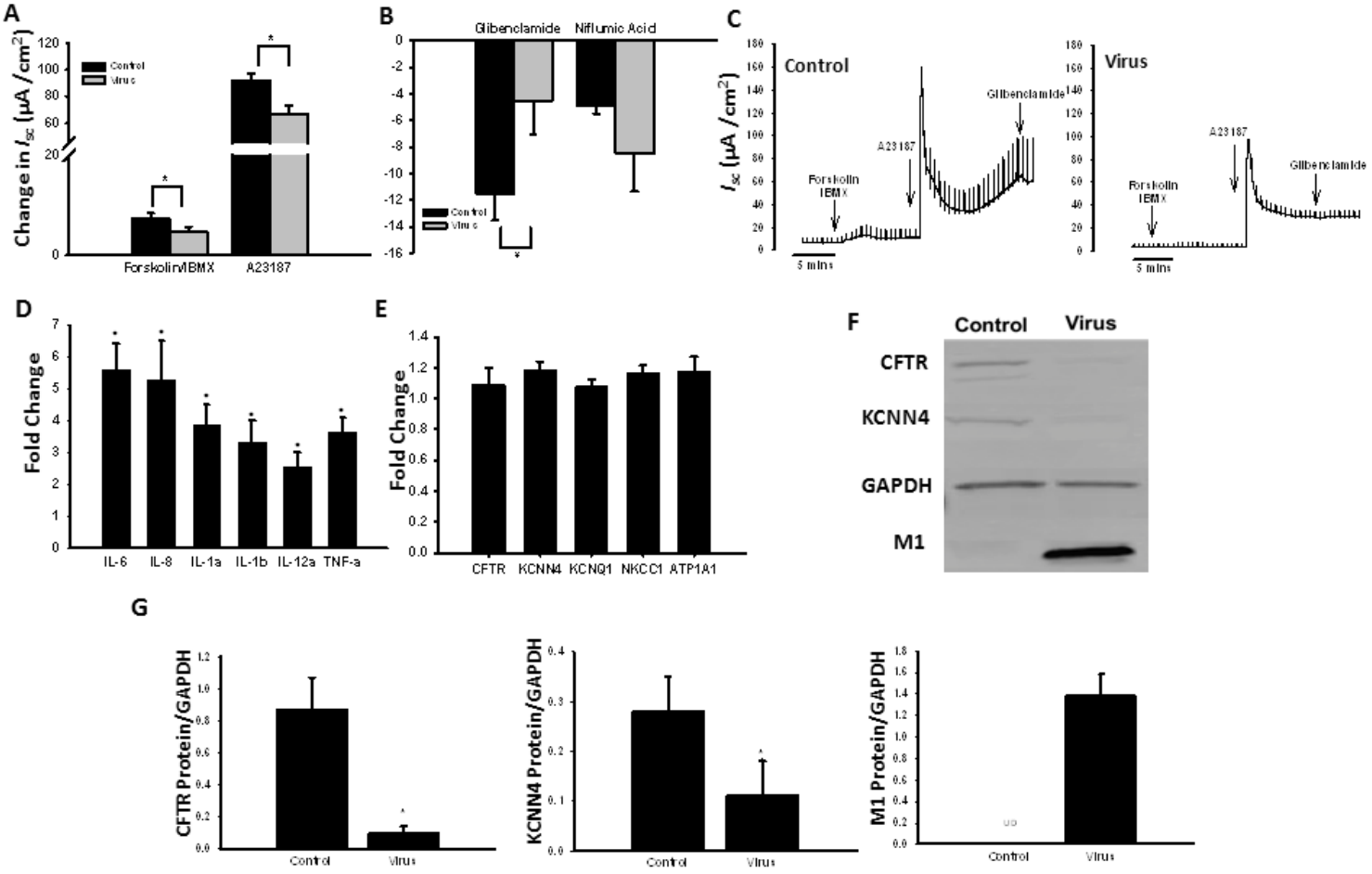

Figure 10. Effect of a high viral dose of influenza (MOI 10) on Calu3 cells 48 hours post-infection.

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A) or inhibitors (glibenclamide and niflumic acid; B) in control and virus-infected cells. Experimental I_{sc} traces representing cellular responses to activating and inhibiting drugs among control and virus-infected cells (C). Change in cytokine mRNA, as measured by RT-qPCR (D). Change in ion channel and ion transporter mRNA, as measured by RT-qPCR (E). Western blot (F) and densitometry (G) of CFTR (~150 kDa), KCNN4 (~45 kDa), and influenza M1 protein (~31 kDa) compared to GAPDH (~37 kDa) reference. I_{sc} = short-circuit current; CFTR = cystic fibrosis transmembrane regulator; NKCC1 = Na^+-K^+-$2Cl^-$ cotransporter; ATP1A1 = Na^+-K^+ ATPase; UD = undetectable; Data presented as mean ± SEM, analyzed using t-test. * = $p < 0.05$

Additionally, we detected a significant increase in IL-6, IL-8, IL-1α, IL-1β, IL-12α and TNF-α cytokine mRNA (Figure 10D), indicating that the virus is capable of eliciting a strong immune response in our cell model. Up-regulation of these, and other inflammatory cytokines, is required to fight the viral infection[46]. However, the intense production of cytokines contributes to the pathogenesis by exacerbating the inflammatory response, which often correlates with increased fluid secretion in the airways[46,48,61,138]. This phenomenon may be a result of both the virus, and subsequent stimulation of the immune response, inducing changes in ion channel expression [48,60,61,117]. Both TNF-α and IL-1β have been directly implicated in impairing fluid clearance in the airways[48,59,117]. It is evident that after 48 hours, at a high MOI, there is a strong up-regulation of pro-inflammatory mediators (Figure 10D). Additionally, it should be noted that in a natural *in vivo* infection, not all of the cells in the airway would become infected[132]. This is important, as discussed by Oh *et al*. [133], in the context of apoptosis, as lower doses of virus result in more non-infected cells that can then be regulated by infected cells in a paracrine fashion. This paracrine cytokine regulation has implications in the development of pulmonary edema [48,59,117]. Therefore, we investigated the impact of a lower dose of virus in our epithelial cell model, using an MOI of 1, or less, to determine whether a particular dose would elicit an immune response that would, in turn, increase secretion.

Interestingly, when the epithelium was only 30% infected (MOI 0.3) we observed no detectable ion transport or epithelial resistance changes 48 hours post-infection (Figure 11A,B). Additionally, this low infectious dose correlated with a

relatively low induction of pro-inflammatory cytokine mRNA (Figure 11G). However, increasing the dose to an MOI of 1, again, resulted in a decrease in cAMP- and calcium-activated I_{sc} (Figure 11E). Additionally, a significant decrease in epithelial resistance of infected cells was detected (119.1 ± 14.3Ωcm^2) compared to non-infected cells (348.2 ± 59.3Ωcm^2). These results are similar to that of the high viral dose (MOI 10; Figure 10A), which would lead to apoptosis and a compromised epithelial barrier. However, an intermediary dose (MOI 0.6) where 60% of the epithelium is infected, a significant increase in the calcium-activated I_{sc} was detected ($p = 0.015$; Figure 11C). Interestingly, with this infectious dose there was no difference in epithelial resistance between infected (189.7 ± 18.1Ωcm^2) and non-infected (229.3 ± 25.1 Ωcm^2) cells. An MOI of 0.6 was able to increase the secretory response without resulting in an increase in the epithelial barrier permeability.

We speculated that the low MOI (0.3) was not sufficient to cause a significant paracrine impact on ion channel physiology because there were no measurable differences in I_{sc} between infected and non-infected cells. The response of the cells infected at an MOI of 1 closely resembled that of the MOI of 10. This lead us to conclude that infecting at an MOI ≥ 1, or under conditions where every cell is infected with virus, that despite any chemokine, paracrine or autocrine regulation there will be a decrease in its secretory response. Results obtained from using the intermediary dose (MOI 0.6) demonstrates that when the entire epithelium is not infected, as would occur during a natural infection, there is an increased secretory response.

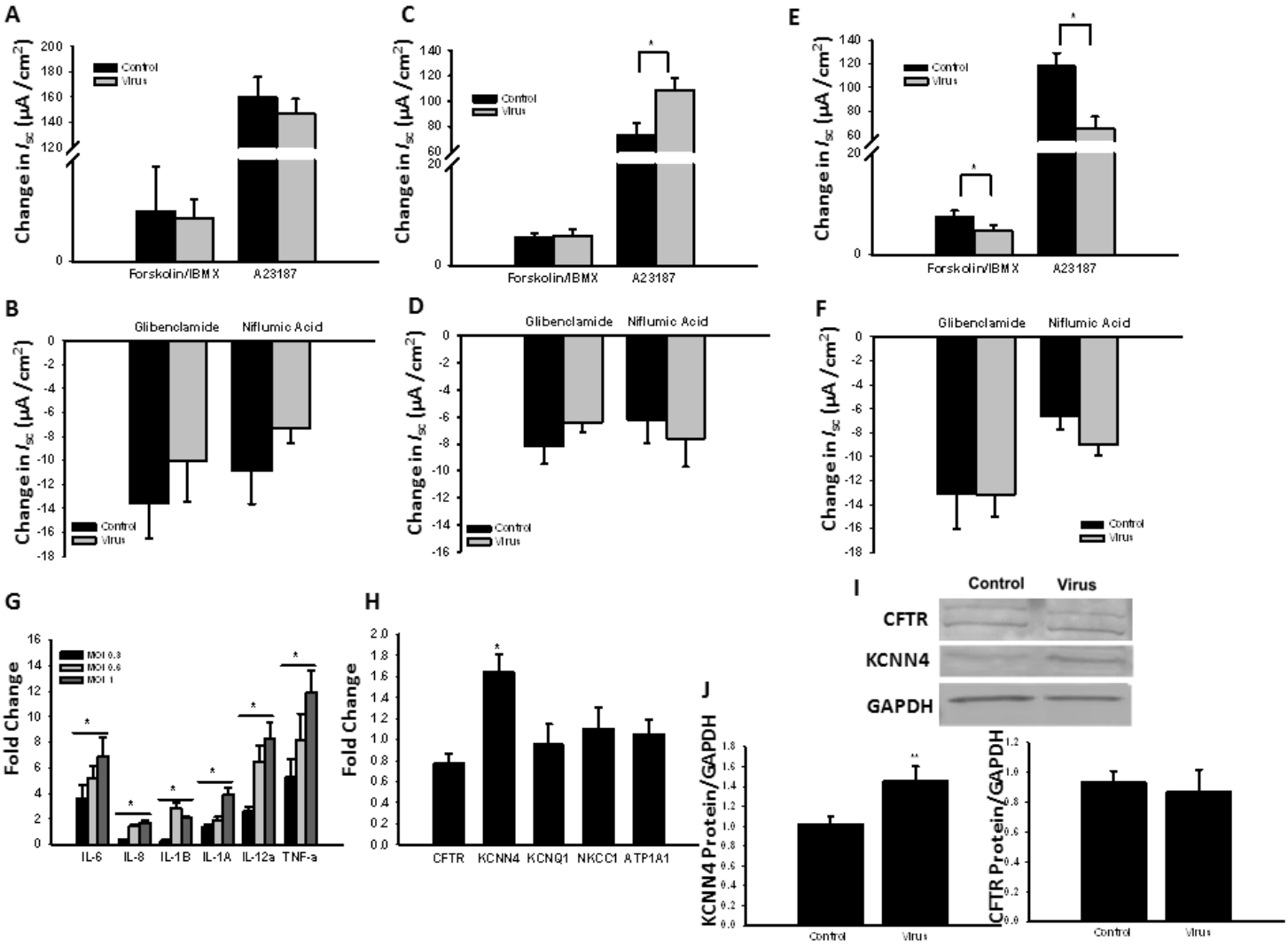

Figure 11. Viral dose response has varying effects on short-circuit current.

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A, C, E) or inhibitors (glibenclamide and niflumic acid; B, D, F) in control and virus-infected cells. Virus-infected cells treated with MOIs of 0.3 (A, B), 0.6 (C, D), and 1.0 (E, F). Change in cytokine mRNA, as measured by RT-qPCR for MOIs 0.3, 0.6 and 1 (G). Change in ion channel and ion transporter mRNA, as measured by RT-qPCR at MOI 0.6 (H). Western blot (I) and densitometry (J) of CFTR (~150 kDa) and KCNN4 (~45 kDa) compared to GAPDH (~37 kDa) reference. MOI = multiplicity of infection; I_{sc} = short-circuit current; CFTR = cystic fibrosis transmembrane regulator; NKCC1 = Na^+-K^+-$2Cl^-$ cotransporter; ATP1A1 = Na^+-K^+ ATPase. Data presented as mean ± SEM, individual experiments analyzed using t-test. * = $p < 0.05$; ** = $p < 0.001$

This resulting secretory phenotype best represents the physiological response to a natural influenza infection.

Analysis of the underlying mechanism driving this secretory response revealed that an MOI of 0.6 correlated with an increase in KCNN4 mRNA ($p = 0.005$; Figure 11H) and protein ($p < 0.001$; Figure 11I, J). KCNN4, a calcium-activated potassium channel, plays a significant role in lowering the membrane potential and driving anion secretion [65]. Up-regulation of this channel, in particular, would result in an increased drive for apical chloride secretion and a greater movement of fluid into the airways. Additionally, up-regulated levels of CFTR could produce the same results; however, analysis at the mRNA and protein level indicates that CFTR is unchanged (Figure 11H, I). Given that we observed a virus dose-dependent up-regulation of inflammatory cytokines (Figure 11G), the partially infected epithelium (i.e., MOI of 0.6) could create an environment with strong paracrine regulation of the non-infected, healthy cells. This paracrine regulation could be driving the up-regulation of KCNN4 at an MOI of 0.6. Such a mechanism, if occurring *in vivo,* would ultimately contribute to the formation of pulmonary edema through an increased driving force for the movement of chloride into the airways.

We confirmed the functional role of KCNN4 by removing its potential contribution to I_{sc} with TRAM-34 (specific inhibitor of KCNN4)[139]. Infected epithelium (MOI 0.6) inhibited with TRAM-34 had significantly reduced calcium-activated I_{sc} compared to non-infected control cultures ($p < 0.001$; Figure 12A). Given that KCNN4 mRNA and protein levels were significantly increased over controls when 60% of the epithelium was infected, we expected to see a greater

degree of inhibition in the infected epithelium. More specifically, inhibition of the KCNN4-associated current significantly decreased the overall I_{sc} by 98% in infected cells, and 91% in non-infected cells (Figure 12A). This study demonstrates that in our cell model when 60% of the epithelium is infected, the driving force for apical chloride secretion is increased, as a result of up-regulated levels of KCNN4. This channel may be only partially responsible for this secretory response, but our data suggest that KCNN4 is the primary channel responsible for the increased calcium-activated I_{sc}. Infecting at an MOI of 0.6, in our model, may mirror what occurs *in vivo* during complicated, acute influenza infection, as not all of the cells become infected and there is potential for paracrine regulation of these non-infected cells. Development of pulmonary edema may be a result from up-regulation of KCNN4, which acts to increase apical anion secretion.

Detection of a secretory response to influenza infection and identification of the key ion channel driving the response may open a new area of pharmacotherapy. Our study is the first to identify KCNN4 as a potential target for drug therapy to reduce the severity of influenza virus infections with regards to pulmonary edema formation. Further mechanistic investigations are required in order to understand the interaction between influenza and KCNN4. However, there is potential for use of clinically available inhibitors to improve the clinical outcomes in patients suffering from severe influenza infections[139,140].

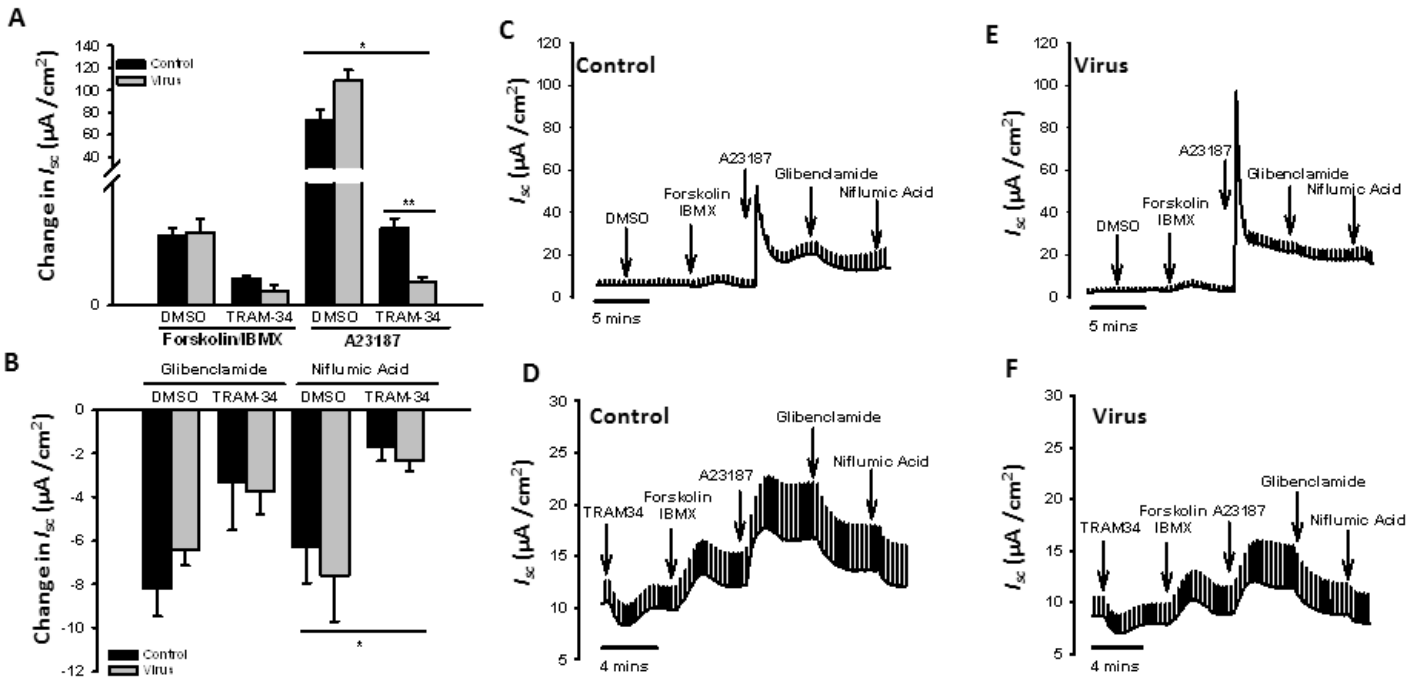

Figure 12. TRAM-34 inhibition reduces calcium-stimulated current in Calu3 cell monolayers infected with influenza virus at MOI 0.6

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A) or inhibitors (glibenclamide and niflumic acid; B) in control and virus-infected cells exposed to the KCNN4 inhibitor TRAM-34 or DMSO control. Experimental I_{sc} traces representing cellular responses to activating and inhibiting drugs among control cells exposed to DMSO control (C) or TRAM-34 inhibitor (D) and virus-infected cells exposed to DMSO control (E) or TRAM-34 inhibitor (F). I_{sc} = short-circuit current. Data presented as mean ± SEM, analyzed using two-way ANOVA with an interaction term. * = $p < 0.05$; ** = $p < 0.001$

CHAPTER 4. RELATIONSHIP BETWEEN MANUSCRIPTS

The first objective was to determine if exposure of Calu3 cells to influenza virus infection for 24 and 48 hours at a high MOI would modulate chloride and/or potassium ion channel function. Our findings in chapter 3 at 24 hours indicate that even at a high viral dose there is no observed change in ion channel function. However, after 48 hours of infection, we did observe significant decreases in both chloride and potassium ion channel function. This finding supports our original hypothesis that a high dose of virus would lead to a decrease in ion channel conductance. Additionally, we determined that using a high dose of virus results in decreased epithelial resistance.

Our secondary objective was to determine a physiologically relevant dose of virus that would be capable of eliciting an immune response sufficient enough to modulate ion channel conductance. Varying the viral dose revealed that when approximately 60% of cells are infected, we observe an increase in secretion. This result is likely due to the up-regulated levels of pro-inflammatory cytokines acting to up-regulate the potassium channel KCNN4.

In order to complete the study and address our remaining objectives, we subjected non-infected cells to a mixture of pro-inflammatory cytokines, so as to model the paracrine regulation independently from the virus. Chapter 5 addresses the results pertaining to specific objectives 3 and 4.

CHAPTER 5. H1N1 DRIVES A PARACRINE REGULATION OF CALU3 CELLS THAT RESULTS IN A HYPERSECRETORY RESPONSE AND INVOLVES A MIXTURE OF PRO-INFLAMMATORY CYTOKINES

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5.1. Abstract

Influenza A virus pathogenesis involves the initial infection of upper airway epithelium and subsequent induction of the host immune response. Initial studies indicate that influenza virus can modulate ion channel function resulting in impairment of mucociliary clearance. This disruption to the fluid balance in the airways leads to pulmonary edema and Acute Respiratory Distress Syndrome (ARDS). Additionally, the strong inflammatory response initiated by viral infection aids in fighting the virus, but has also been shown to further modulate ion channel transport. Here, we have described the effect of cytokine-containing conditioned media (CM) on non-infected, polarized Calu3 cell monolayers. This mirrors the situation observed during influenza infections where the majority of cells are not infected with the virus, but are exposed to the paracrine effects of inflammatory cytokine mediators from the infected cells. Treatment with CM resulted in an increased secretory phenotype after 48 hours ($p < 0.001$). The chloride and potassium channel function was measured by means of a short-circuit current (I_{sc}) produced by the Calu3 cell monolayer in an Ussing chamber. We investigated whether paracrine regulation was responsible for this secretory phenotype, and not the virus infection itself, as modeled with the CM. Interestingly, we found that the secretory phenotype induced by the CM treatment was a result of concerted up-regulation of KCNN4 and KCNQ1. These potassium channels are responsible for generating the driving force for apical chloride secretion. Specific inhibition of these channels ameliorated the response, further indicating their importance in driving fluid secretion. Additionally, previous studies have shown that individual cytokines

may have the capacity to modulate ion channel expression in airway epithelium; therefore, we dosed cells with IL-8 in order to assess whether, or not, this particular pro-inflammatory cytokine would be able to reproduce the same effect as the conditioned media. IL-8 treatment did result in an increased secretory response; however, the underlying mechanism driving is not mediated through up-regulated KCNN4 and KCNQ1.

5.2. Introduction

On an annual basis, influenza A virus is responsible for over half a million deaths due to complications related to mucus and fluid clearance[8]. Appropriate fluid clearance from the lungs is essential for adequate gas exchange[31]. Disruption of the mechanism driving fluid clearance results in the formation of pulmonary edema[33]. This is particularly salient in severe infections where the inflammatory response initiated by infection of the upper airway epithelial cells quickly spreads to the lower airways, resulting in influenza-induced Acute Respiratory Distress Syndrome (ARDS)[7,35]. The ARDS is a result of a massive cascade of cytokines, both pro- and anti-inflammatory, flooding into the airways [48,107,108]. Although the virus does not infect the majority of the cells in the airways, this cytokine cascade affects all of the epithelial cells, which control the movement of fluid[132]. Upon infection with influenza, the airway epithelial cells, along with inflammatory cells (e.g., macrophages), generate a large proportion of the cascade[10,13]. Individually, a number of these cytokines have been shown to impact epithelial ion transport; however, the combined effects that would arise during an influenza infection, have not been investigated[48,107,108,109].

Ion transport across the epithelial barrier creates the osmotic drive to control fluid movement in and out of the airways[11,28,130]. The driving force for movement of ions and fluid in all epithelial cells is generated by the electrochemical gradient of the $3Na^+/2K^+$-ATPase pump (ATP1A1)[11]. This creates a negative internal potential: high internal potassium concentration paired with a low concentration of sodium[21,104]. The negative membrane potential drives chloride out of the cell apically, pulling sodium and water along with it[104]. The majority of this apical anion secretion is through the cAMP-activated cystic fibrosis transmembrane regulator channel, CFTR[22,97,141]. Chloride movement is also facilitated through the TMEM16 family of calcium-activated chloride channels (CaCC)[24,25,142]. The electrochemical gradient driving chloride out through these channels leads to airway hydration. The chloride lost apically is subsequently replenished on the basolateral side through the Na^+-K^+-$2Cl^-$ co-transporter (NKCC1). This net secretion can be increased by activation of potassium channels, which facilitate K^+ efflux, which further depolarizes the membrane and increases chloride secretion. [69,143,144]. Airway epithelial cells have two major potassium channels, namely KCNN4 (calcium-activated), and KCNQ1 (cAMP-activated)[89,100].

Previously, we have shown that if every cell is infected with influenza virus, we observe a decrease in net secretion after 48 hours of infection (see chapter 3; Figure 10 A, B). This was a result of down-regulation of CFTR, and KCNN4 protein (Figure 10F). However, if we reduce the viral dose, recapitulating the situation in the airways where not all of the cells are infected, there is an observed increase in net secretion primarily driven through KCNN4 (see chapter 3; Figure 11C, I). This

effect also correlated with an increase in the relative transcript levels for various pro-inflammatory cytokines, including IL-6, IL-8, IL-1β, IL-1α, IL-12α and TNF-α, which are secreted by the infected epithelial cells (Figure 11G). The majority of this response is pro-inflammatory; however, we speculate that the observed increase in fluid secretion is driven by a combination of these cytokines acting in a paracrine manner on non-infected cells. This mirrors what occurs *in vivo*, as the massive fluid secretion leading to ARDS is a result of a cytokine cascade that presumably includes multiple different inflammatory mediators[115,145,146]. In this study, we wanted to assess the impact of conditioned media (CM) containing these cytokines on Calu3 cell fluid secretion. This model allows us to study the impact of the inflammatory cytokines in the absence of the virus. Additionally, we test whether an individual pro-inflammatory cytokine, IL-8, can independently mediate a secretory response.

5.3. Material and Methods

Refer to Chapter 2

5.4. Results

Here we exposed polarized Calu3 cells to conditioned media (CM), which contains the secreted inflammatory mediators induced by influenza virus infection of epithelial cells. The goal was to assess whether the release of cytokines from infected cells would be capable of paracrine stimulation of non-infected cells, leading to a secretory phenotype. Polarized Calu3 cells were exposed both apically and basolaterally with UV-inactivated viral conditioned media (VCM; see chapter 2 section 2.3.) or control conditioned media (CCM) for 48 hours. This time point was

selected in order to be comparable to our previous data where we saw an increased secretory response in cells infected at an MOI of 0.6 for 48 hours (Figure 11C).

We found that the cells treated for 48 hours with VCM had significantly increased calcium-activated I_{sc} ($p < 0.001$) with no change in cAMP-activated I_{sc} when compared to controls (Figure 13A). Additionally, no change in measurable I_{sc} for cAMP- or calcium-activated chloride conductance was detected after inhibition with glibenclamide or niflumic acid, respectively (Figure 13B). A possible mechanism to explain this increased driving force for apical chloride secretion includes up-regulated levels of either NKCC1 and/or ATP1A1. However, mRNA levels of both of these transporters were not significantly increased (Figure 13D). Alternatively, increased chloride conductance through CFTR could account for the observed effect; however, the mRNA levels of CFTR remained unchanged (Figure 13D). Further molecular analysis revealed that there was a significant 2-fold increase in KCNN4 and KCNQ1 mRNA in the VCM treated cells ($p = 0.039$; $p = 0.017$, respectively; Figure 13D). This correlated with a significant increase in KCNN4 protein ($p = 0.002$; Figure 13F), giving a plausible explanation for the observed increase in calcium-activated I_{sc}. With no difference in the KCNQ1-stimulated I_{sc}, as indicated by addition of forskolin and IBMX, we assessed whether the channel subunits, KCNE2 and KCNE3, were present. Both subunits are essential in maintaining a cAMP-stimulated I_{sc}[89]; however, neither one was detectable via RT-qPCR, despite using previously published primers[65], and multiple attempts using newly designed primers. Additionally, the KCNQ1 channel protein was below

detectable limits when using western blotting techniques. This is a possible explanation for detecting no difference in the cAMP-stimulated I_{sc}.

In order to assess whether pro-inflammatory cytokines may be responsible for driving this increased secretory response, we quantified and compared cytokine protein levels in the VCM relative to CCM using the Bio-Plex suspension array assay. Cells treated with VCM were exposed to a strong, pro-inflammatory cytokine mixture, with elevated levels of IL-6, IL-8, and TNF-α ($p < 0.001$ for all; Figure 13E).

With levels of KCNQ1 mRNA increased, but without detectable protein, we wanted to establish whether or not KCNQ1 was present in our cell model due to previous reports; therefore, we inhibited its potential contribution to I_{sc} with chromanol 293B (Figure 14)[147]. Expectedly, chromanol 293B inhibition decreased the cAMP-mediated I_{sc} by 59% ($p = 0.07$; Figure 14A). Additionally, there was a significant decrease in the glibenclamide-inhibitable I_{sc} ($p = 0.029$) suggesting that cAMP-mediated chloride secretion depends on KCNQ1 potassium conductance (Figure 14B). Surprisingly, chromanol 293B inhibition also resulted in a significant decrease in calcium-activated I_{sc} ($p = 0.003$; Figure 14A). This suggests that the increase in apical calcium-activated chloride secretion is dependent on KCNQ1. We speculated that both KCNQ1 and KCNN4 are required in order to see the overall net increase in anion secretion, as observed after treatment with conditioned media (Figure 13A).

We then sought to investigate the physiological contribution of KCNN4 to the I_{sc} in conditioned media treated cells using the channel specific inhibitor TRAM-34.

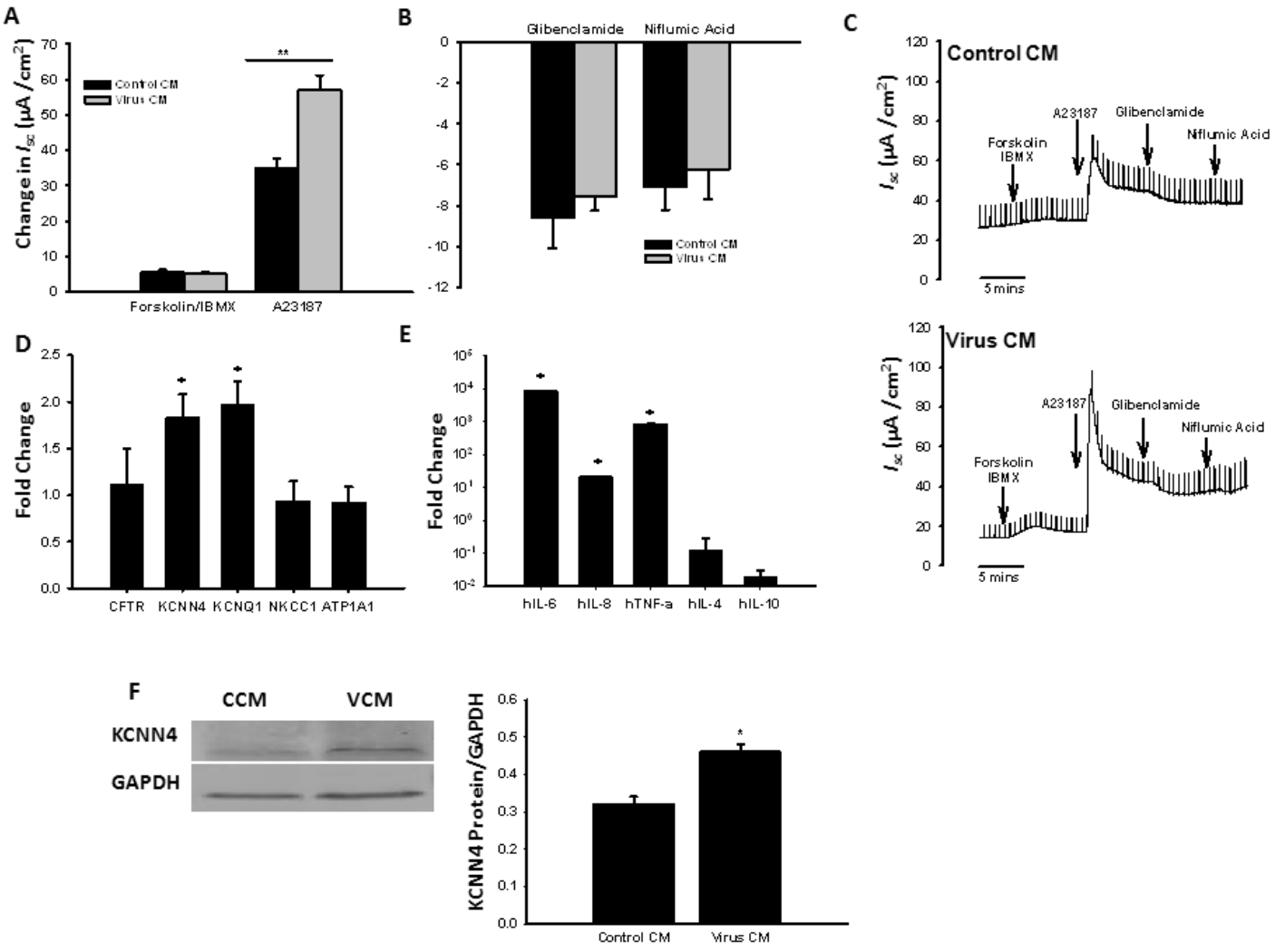

Figure 13. 48 hour CM treatment causes increase in calcium-activated short-circuit current.

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A) or inhibitors (glibenclamide and niflumic acid; B) in control CM and virus CM. Experimental I_{sc} traces representing cellular responses to activating and inhibiting drugs in control CM and virus CM (C). Change in ion channel and ion transporter mRNA, as measured by RT-qPCR (D). Change in cytokine mRNA, as measured by RT-qPCR (E). Western blot and densitometry of KCNN4 (~45 kDa) compared to GAPDH (~37 kDa) reference (F). I_{sc} = short-circuit current; CM = conditioned media. Data presented as mean ± SEM, analyzed using t-test. * = $p < 0.05$; ** = $p < 0.001$

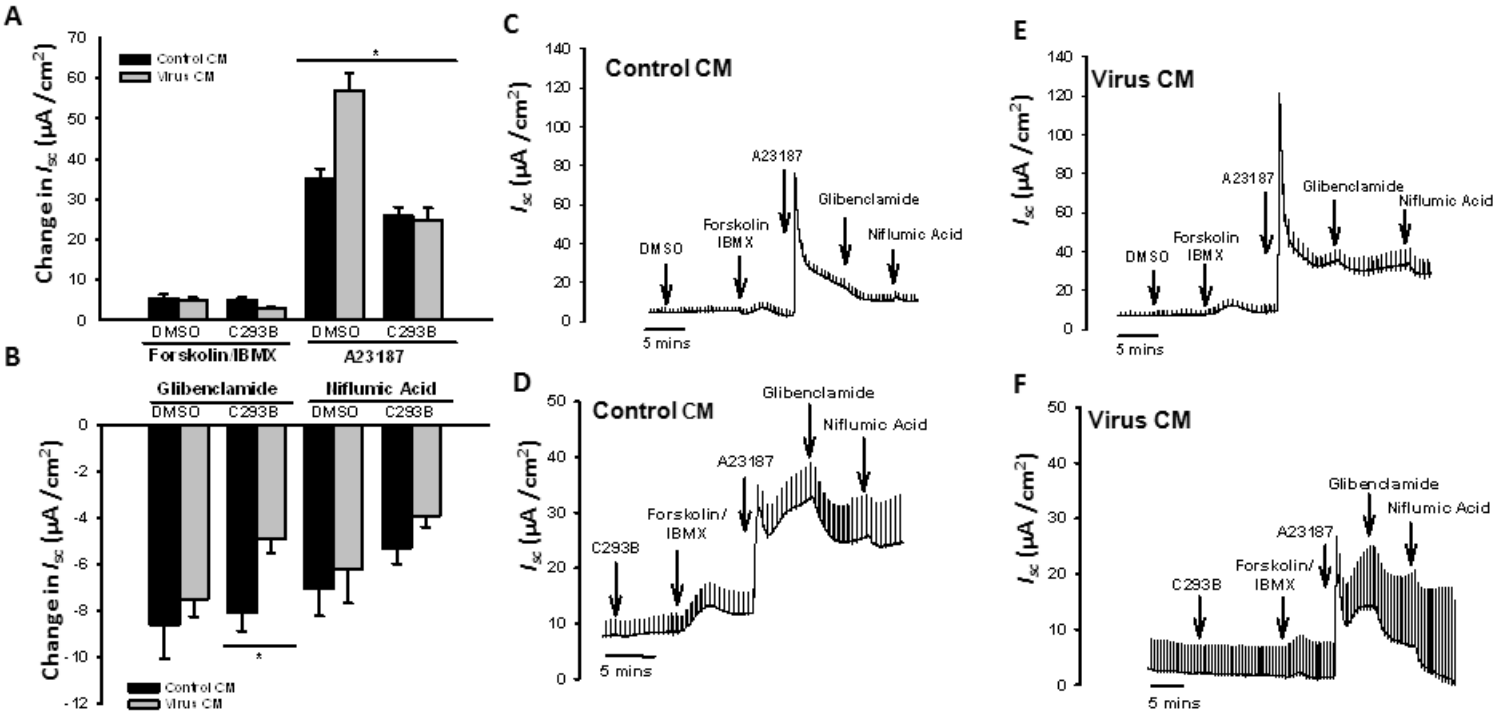

Figure 14. Chromanol 293B inhibition reduces calcium-activated current in CM treated cells.

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A) or inhibitors (glibenclamide and niflumic acid; B) in control CM and virus CM exposed to the KCNQ1 inhibitor chromanol 293B or DMSO control. Experimental I_{sc} traces representing cellular responses to activating and inhibiting drugs among control CM cells exposed to DMSO control (C) or chromanol 293B inhibitor (D) and virus CM cells exposed to DMSO control (E) or chromanol 293B inhibitor (F). I_{sc} = short-circuit current; CM = conditioned media. Data presented as mean ± SEM, analyzed using two-way ANOVA with an interaction term. * = $p < 0.05$

Calcium-activated I_{sc} was significantly decreased upon inhibition, as expected ($p < 0.001$), to a level that was equivalent between VCM and CCM treated cells (Figure 15A). Inhibition of KCNN4 will decrease the overall potassium-related driving force on the basolateral membrane, thereby reducing the inhibitable CFTR-mediated chloride current. We observed a decrease in the chloride current of approximately two-thirds upon inhibition with glibenclamide ($p < 0.001$; Figure 15B). A minimal decrease was observed with niflumic acid, as there appeared to be less inhibitable chloride current relative to DMSO control cells (Figure 15B).

To further investigate the contribution of KCNN4, we utilized a more selective and potent inhibitor of this channel (ICA-17043)[139,148]. Inhibition with ICA-17043 significantly reduced the overall cAMP-activated I_{sc} ($p = 0.004$; Figure 16A). Correspondingly, the glibenclamide-inhibitable current was significantly decreased, due to a reduced driving force ($p = 0.007$; Figure 16B). The calcium-activated I_{sc} in VCM treated cells was inhibited to a level below the CCM treated cells ($p = 0.019$), with the overall reduction being 94% and 84%, respectively ($p < 0.001$; Figure 16A).

Interestingly, the niflumic acid-inhibitable current was larger in VCM treated cells than CCM treated cells ($p = 0.01$; Figure 16B). We speculate that this difference may be a result of the KCNQ1-related potassium conductance from the basolateral membrane, as KCNQ1 has been shown to have some basolateral localization, and is up-regulated in the VCM treated cells[89,91]. This would maintain the driving force for apically secreted chloride in the absence of KCNN4, and result in an increase in inhibitable current by niflumic acid in VCM treated cells. Therefore, we believe that

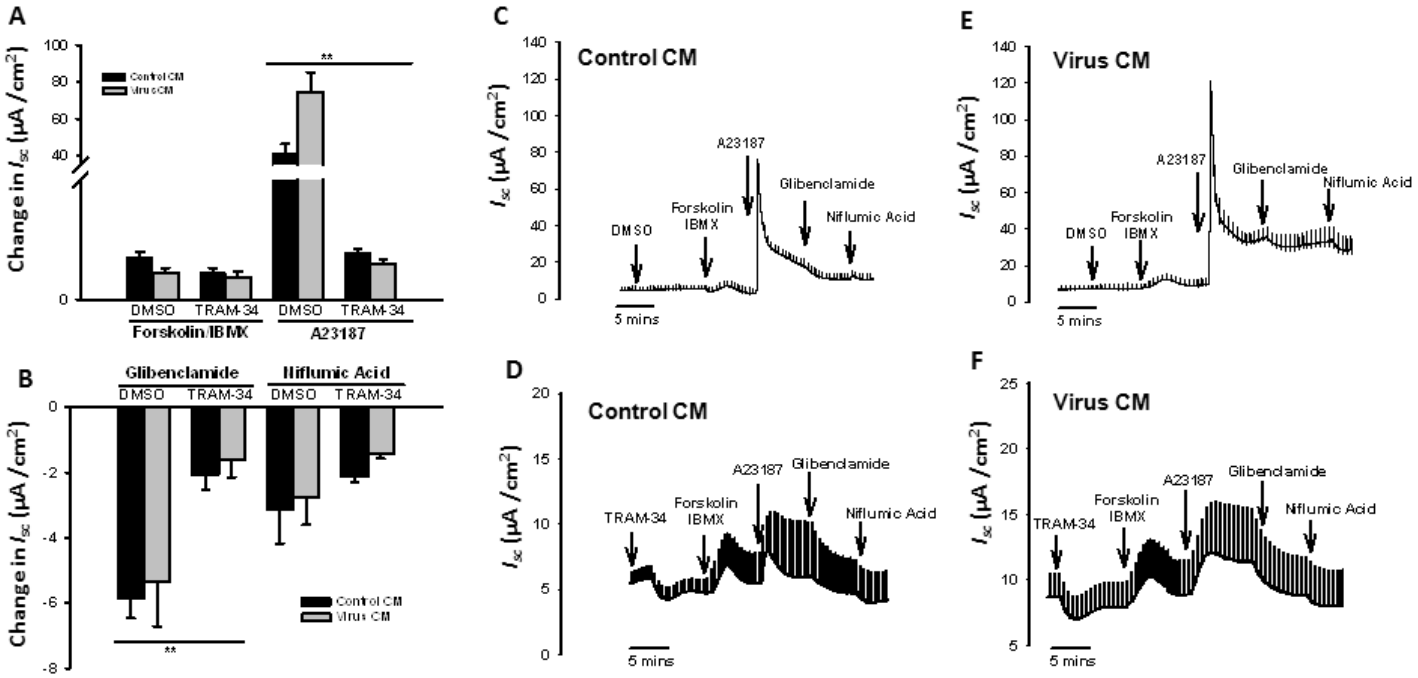

Figure 15. TRAM-34 inhibition of CM treated cells reduced the calcium-activated short-circuit current.

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A) or inhibitors (glibenclamide and niflumic acid; B) in control CM and virus CM cells exposed to the KCNN4 inhibitor TRAM-34 or DMSO control. Experimental I_{sc} traces representing cellular responses to activating and inhibiting drugs among control CM cells exposed to DMSO control (C) or TRAM-34 inhibitor (D) and virus CM cells exposed to DMSO control (E) or TRAM-34 inhibitor (F). I_{sc} = short-circuit current; CM = conditioned media. Data presented as mean ± SEM, analyzed using two-way ANOVA with an interaction term. ** = $p < 0.001$

basolaterally-located KCNQ1 has a marginal contribution to the apical chloride secretion, with complete inhibition of KCNN4.

Interleukin-8 is a strong, pro-inflammatory cytokine that is highly expressed by epithelial cells during viral infection[56,106]. Since the direct effects of IL-8 have not yet been studied in epithelial cells, we sought to characterize the role that this single, pro-inflammatory cytokine has in modulating anion secretion in Calu3 cells, using a dose-response (low (10), medium (100) and high (1000) ng/ml).

Treatment of cells for 48 hours with IL-8 produced a strong pro-secretory response as evidenced by a significantly increased calcium-activated I_{sc} at low, medium and high doses ($p < 0.001$, 0.012, 0.002, respectively; Figure 17A). There was no change in the cAMP-stimulated I_{sc} for any of the doses of IL-8. However, the high dose resulted in a significant reduction in inhibitable chloride conductance, for both glibenclamide ($p = 0.002$) and niflumic acid ($p = 0.011$; Figure 17B). This decrease in chloride conductance is likely a result of a significant decrease in the basolateral supply of chloride into the cell by NKCC1 and not due to a decrease in CFTR, as no change was noted in the mRNA (Figure 17D). Additionally, RT-qPCR analysis for the other ion channels under study showed no change in mRNA; however, there was a significant decrease in KCNN4 mRNA after treatment with the high dose of IL-8 (Figure 17D). Interestingly, the low dose of IL-8 increased mRNA levels of the pro-inflammatory cytokines IFN-α, TNF-α, IL-1β and IL-1α (Figure 17E). This demonstrates that IL-8, by itself, is not able to recapitulate the cellular

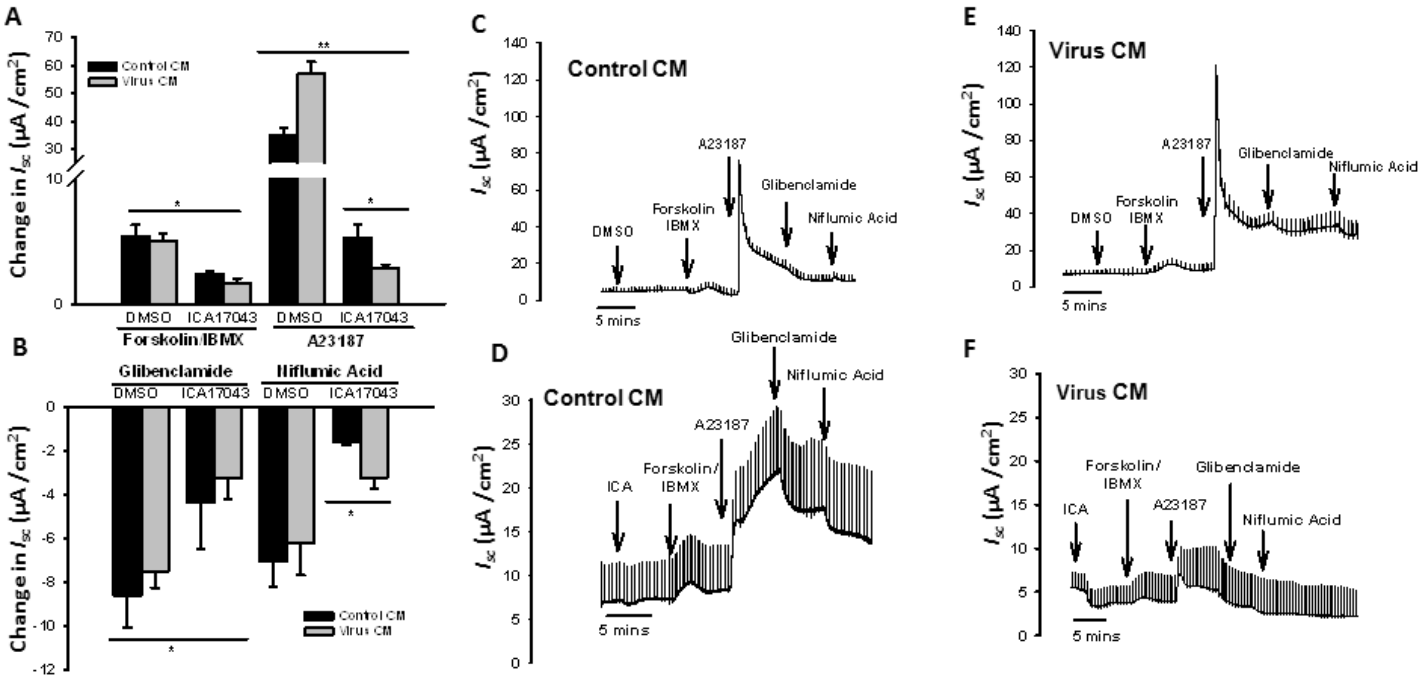

Figure 16. ICA-17043 inhibition reduces overall short-circuit current in CM treated cells.

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A) or inhibitors (glibenclamide and niflumic acid; B) in control CM and virus CM cells exposed to the KCNN4 inhibitor ICA-17043 or DMSO control. Experimental I_{sc} traces representing cellular responses to activating and inhibiting drugs among control CM cells exposed to DMSO control (C) or ICA-17043 inhibitor (D) and virus CM cells exposed to DMSO control (E) or TRAM-34 inhibitor (F). I_{sc} = short-circuit current; CM = conditioned media. Data presented as mean ± SEM, analyzed using two-way ANOVA with an interaction term. * = $p < 0.05$; ** = $p < 0.001$

physiological effects of the conditioned media, which contains multiple different cytokines.

5.5. Discussion

Calu3 cells treated with VCM for 48 hours produced a robust secretory response (Figure 13A). Molecular analysis of mRNA and protein indicates that KCNN4 is up-regulated, which is in agreement with our previous study (see chapter 3). A plausible mechanism for the increased secretory response is the up-regulation of NKCC1 and/or ATP1A1. The Na^+-K^+-ATPase (ATP1A1) creates the electrochemical gradient for the apical movement of chloride and up-regulation of this pump would result in an increased driving force for chloride secretion[11]. Similarly, the Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) replenishes intracellular stores of these ions, so any increase in activity would result in more driving force for apical secretion[11]. Since we did not detect changes in expression of either of these transporters it is unlikely that they are responsible for driving the increased fluid secretion. Therefore, we further investigated the contribution of KCNN4 to this process.

Using two selective inhibitors of KCNN4, we demonstrated that the increase in apical chloride secretion could be ameliorated. TRAM-34 significantly decreased the calcium-activated I_{sc} and the more potent inhibitor, ICA-17043 showed further reduction in the I_{sc} to levels below the CCM treated cells.

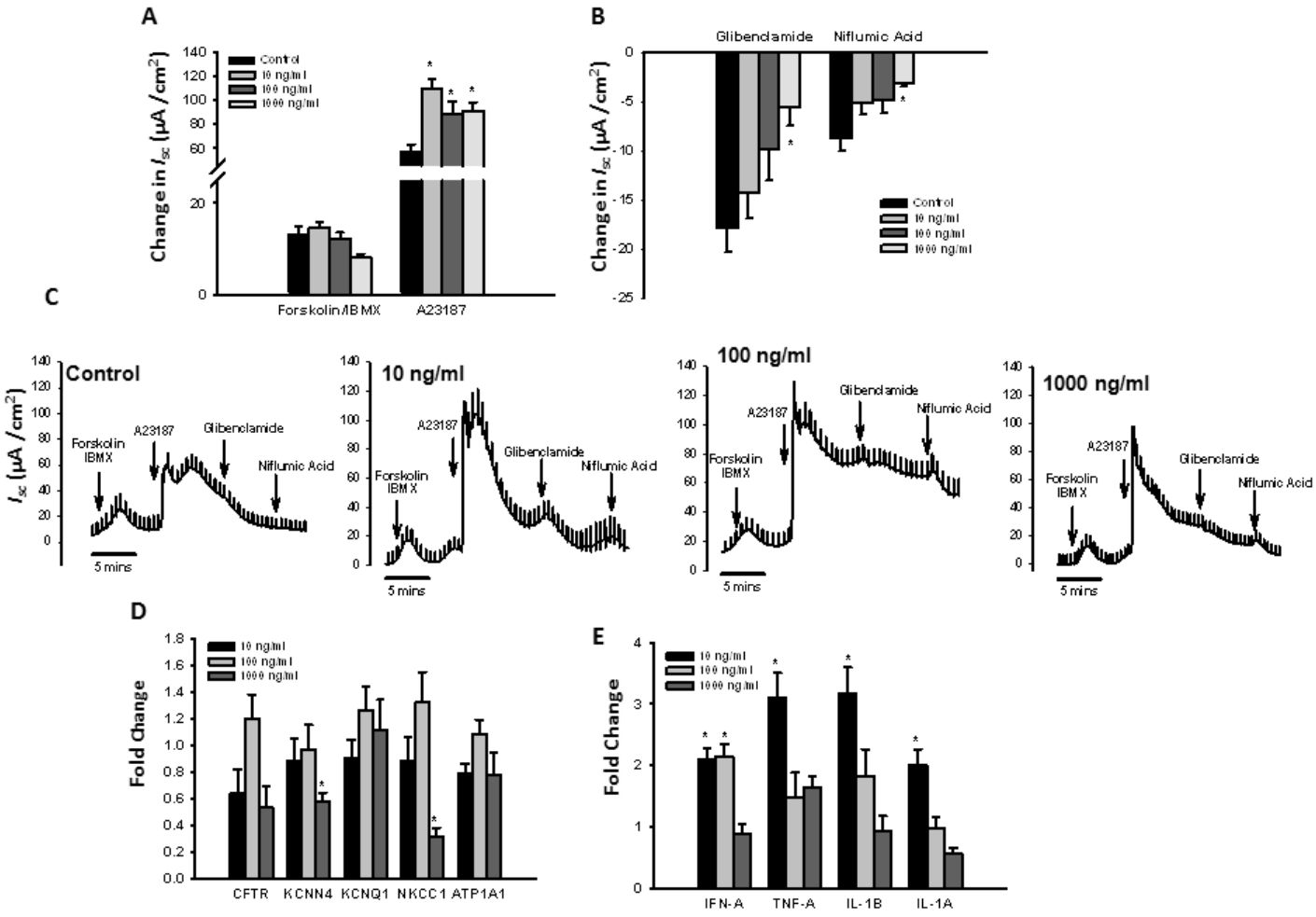

Figure 17. IL-8 dose response leads to physiological increase in calcium-activated short-circuit current.

Change in I_{sc} in response to the addition of ion channel activators (forskolin/IBMX and A23187; A) or inhibitors (glibenclamide and niflumic acid; B) in cells treated with IL-8. Experimental I_{sc} traces representing cellular responses to activating and inhibiting drugs in cells treated with IL-8 (C). Change in ion channel and ion transporter mRNA, as measured by RT-qPCR (D). Change in cytokine mRNA, as measured by RT-qPCR (E). I_{sc} = short-circuit current; CFTR = cystic fibrosis transmembrane regulator; NKCC1 = Na^+-K^+-$2Cl^-$ cotransporter; ATP1A1 = Na^+-K^+ ATPase. Data presented as mean ± SEM. T-tests were used to compare control cells to each dose of IL-8. To protect against an inflated type I error rate due to multiple comparisons, the Bonferroni correction was used to reduce the individual test alpha level to 0.0167; * = $p < 0.05$

The role of KCNN4 is to establish the driving force for apical chloride secretion[65]. Therefore, our ability to inhibit KCNN4 indicates that the increased secretory response in VCM treated cells is closely linked to increases in KCNN4 expression.

KCNN4, also referred to as the Gardos channel, is a calcium-activated potassium channel found in red blood cells, and multiple different tissues[139,148,149,150]. In epithelial cells, it is primarily found on the basolateral membrane, and is highly expressed in Calu3 cells[63,65]. Activation of KCNN4 is mediated through increases in intracellular calcium levels resulting in an increased driving force for apical chloride secretion[63,69]. Previously it was believed that the basolateral recycling of potassium through KCNN4 and the Na^+-K^+-ATPase was solely responsible for driving apical anion secretion in the upper airways[100,101]. However, this model has been updated to include an apical potassium conductance in addition to basolateral conductance[87,89,104].

KCNQ1 is a cAMP-activated potassium channel that is highly expressed in cardiac tissue and various epithelium[151]. In Calu3 cells, KCNQ1 has been identified as the major apical potassium channel, with some basolateral localization[65,89]. In response to VCM treatment, we detected increased mRNA levels of KCNQ1. A limitation is that we were unable to detect the associated protein via western blot despite using three different protein extraction methods, and two different primary antibodies, one of which had been previously shown to work in a Calu3 cell model[89]. To address this, we used the KCNQ1-specific inhibitor, chromanol 293B[89,147] to eliminate the potential contribution of KCNQ1 to the overall I_{sc}. Successful reduction in the calcium-activated I_{sc} indicates that KCNQ1 can

be inhibited pharmacologically. With both physiology and gene expression data in support of the presence of KCNQ1, we sought to identify its associated subunits KCNE2 and KCNE3. Several attempts were made to detect both KCNE2 and KCNE3 in our model using RT-qPCR techniques; however, we were unable to successfully detect the presence of these subunits despite using our own primers and previously published primers[65]. This negative result may explain why stimulation with forskolin and IBMX did not result in an increased cAMP-activated I_{sc}, despite increased levels of KCNQ1 mRNA (Figure 13A), as Moser *et al*. describes that association of KCNQ1 with its subunits allows for the generation of a cAMP-stimulated current[89]. Additionally, the likely presence of KCNQ1 helps to explain the observations from the inhibition of KCNN4 using ICA-17043 (Figure 16B). We observed that after removing the effect of KCNN4, there was a remaining, niflumic acid-inhibitable current in the VCM treated cells. This could be attributed to basolaterally localized KCNQ1, as this may provide enough driving force, along with the up-regulation, to increase chloride secretion in the absence of KCNN4.

Calu3 cells have already been shown to express functional potassium channels[65,87,89]. Our data supports the presence of both KCNN4 and KCNQ1, which are major contributors to the overall driving force for apical anion secretion[65,89]. Located on the basolateral membrane, KCNN4 is responsible for recycling potassium back out of the cell in response to the active pumping of the Na^+-K^+-ATPase[100]. Apically, KCNQ1 works in a similar fashion to drive potassium out of the cell[89]. The combined effect of these two channels creates the driving force for apical chloride secretion via CFTR. The increase in potassium channels

induced by the VCM therefore results in more chloride secretion and the development of a secretory phenotype. Our data further suggests that the secretory response can be ameliorated upon channel-specific inhibition. However, both channels are required in order to observe the secretory phenotype (Figure 18). This agrees with the theoretical model of Cook *et al*., which describes the presence of an apical potassium channel in concert with a basolateral potassium conductance in order to facilitate optimal conditions for the apical secretion of chloride[104]. The apical potassium efflux maintains hyperpolarizing conditions at the apical membrane and reduces the likelihood that chloride will reach its equilibrium gradient; therefore, maintaining a net apical secretion of anions[89,104].

Our results demonstrate, so far, that the pro-inflammatory cytokine mixture is capable of driving increased I_{sc}, which is dependent on the combined up-regulation of KCNQ1 and KCNN4. Studies have shown that prolonged exposure to certain pro-inflammatory cytokines, such as TNF-α and IL-1β, cause a decrease in the absorptive capacity of the epithelium[48,58,59]. Additionally, high doses of anti-inflammatory cytokines, including IL-4 and IL-13, lead to increased chloride secretion through up-regulation of CFTR, and CaCC [107,108]. However, individual cytokines act on a variety of cellular receptors and often initiate different signal transduction pathways[152]. For instance, anti-inflammatory cytokines such as IL-4 result in dampening of the immune response and tissue repair whereas IL-8, a pro-inflammatory cytokine, leads to immune cell recruitment and heightening of the inflammatory response[106,108]

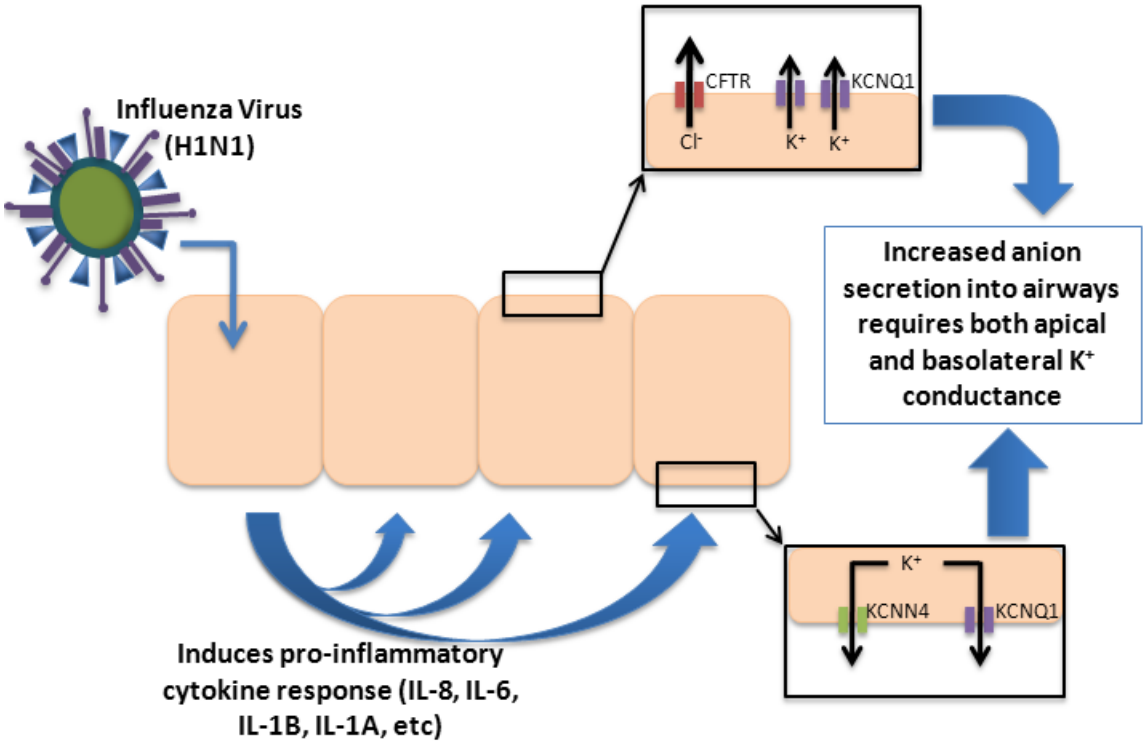

Figure 18. Proposed mechanism of changes in short-circuit current resulting from pro-inflammatory paracrine signaling among non-infected airway epithelial cells.

Influenza A virus infection of the upper respiratory tract leads to the production and secretion of pro-inflammatory cytokines. Many different cytokines are released by the infected epithelium, and since not all of the cells are infected, these immune mediating cytokines act in a paracrine fashion on non-infected cells. We have detected, using a mixture of cytokines on non-infected cells (i.e., the conditioned media) an up-regulation of KCNN4 mRNA and protein, as well as increased KCNQ1 mRNA expression. The outward movement of potassium hyperpolarizes the cell, which increases the driving force for anion secretion (chloride). Up-regulation of these channels results in an increased driving force for chloride efflux through CFTR, thereby increasing the overall fluid secretion into the airways. The presence of both apical and basolateral potassium conductance is necessary in order to generate enough driving force to increase apical anion secretion.

Other cytokines, such as IL-6, which seem to sit in the middle of this spectrum, can mediate both a pro- and anti-inflammatory response[56]. This demonstrates that cytokines may have varying effects depending on whether they are acting independently, or in concert with one another.

The pro-inflammatory cytokine, IL-8, is ubiquitously and consistently secreted upon infection with varying strains of influenza virus, making it an ideal candidate for assessing whether a single pro-inflammatory cytokine is capable of inducing the same response as the CM[56,106,115,153]. Additionally, the direct effects of IL-8 have not yet been studied in epithelial cells even though it is a key cytokine involved in immune regulation and inflammation[56,106,115]. A 48-hour dose response of IL-8 on Calu3 cells resulted in an increased calcium-activated I_{sc}, similar to the conditioned media (Figure 17A). In contrast to the conditioned media, IL-8 treatment did not result in up-regulation of KCNN4 and KCNQ1, indicating that the IL-8-induced hypersecretory phenotype is mediated through a separate mechanism. In fact, KCNN4 and NKCC1 mRNA levels were down-regulated, but only at the high dose of IL-8. This drastic difference between the IL-8 treated cells and the VCM treated cells highlights the inadequacy of reducing a complex, paracrine regulation system down to a single contributing factor. Additionally, the IL-8 results varied by dose, suggesting that physiological changes may be influenced by cytokine concentration. These observations imply that paracrine regulation of epithelial cells depends on both the presence of multiple different cytokines, as well as their relative concentrations. Future research could explore the possible interaction between cytokines and their effect on cell secretory behavior; however, this is

beyond the scope of this study.

Additional applications that could be considered by future research include treatment of influenza-induced ARDS with the clinically available drug, ICA-17043[139]. This compound is currently in phase 3 clinical trials for the treatment of sickle cell anemia[148,149]. Since the underlying mechanism causing cell sickling is dependent on KCNN4 (the Gardos channel) there may be additional therapeutic benefits of ICA-17043 in treatment of acute flu. Overall, our data suggest that the pro-inflammatory cytokines secreted by infected epithelial cells are capable of inducing a secretory response independently of the virus. The concerted up-regulation of KCNN4 and KCNQ1 after CM treatment indicates that both of these channels are required to increase apical chloride secretion. IL-8 treatment resulted in a similar increase in secretory phenotype; however, the underlying mechanism remains to be uncovered, as it is not a result of increased KCNN4 or KCNQ1. This study reveals the impact cytokine expression levels have on airway epithelial cell secretion, and strongly suggest a role for the host immune response in driving the pathogenesis of influenza virus.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSION

The main purpose of this study was to identify the direct and indirect effects of influenza virus infection on chloride and potassium channel function. In order to study the virus-specific effects, we infected Calu3 cells directly at varying doses and assessed changes in ion transport in addition to measuring the immune response (Objectives 1 and 2). The indirect effects refer to the secondary impact of inflammatory cytokines, which are secreted by the infected cells and act in a paracrine manner to modulate ion transport in non-infected cells. We assessed the impact of these inflammatory mediators independently from the virus by using cytokine-containing conditioned media (Objectives 3 and 4). Understanding what drives the pathogenesis of influenza is important in treating severe infections where the patient experiences flooding of fluid into the airways.

Infection with influenza can lead to the development of ARDS and pulmonary edema[5,55,56]. The mechanism driving this secretory response is unclear. The results presented here indicate that the immune response elicited by influenza infection lead to an up-regulation of potassium channels, which drive anion secretion. Chapter 3 described the direct effects of viral infection on polarized Calu3 cell monolayers. We found there was a strong pro-inflammatory cytokine response from cells infected at an MOI of 10, which also correlated with decreased epithelial resistance. This situation closely resembles that of severe influenza infections where an increased viral load overwhelms the host, resulting in cellular apoptosis and flooding of the lungs[154]. More importantly, we determined a physiologically

relevant dose of virus, which allowed us to study both direct and indirect (i.e., cytokines) effects of influenza infection.

The indirect effects of influenza infection were described in Chapter 5 where we studied the effect of cytokine-containing conditioned media on non-infected, polarized Calu3 cell monolayers. This mirrors the situation observed during influenza infections where the majority of cells are not infected with the virus, but they are all exposed to the paracrine effects of inflammatory cytokine mediators from the infected cells[132,133]. Using an MOI of 0.6 we demonstrated that when a large proportion of cells are not infected, but exposed to paracrine regulation from infected cells, the epithelium as a whole develops a secretory phenotype. Based on the analysis of mRNA and protein we concluded this effect to be driven mainly by increased basolateral potassium conductance through up-regulation of KCNN4 (see chapter 3). Chapter 5 investigated whether the paracrine regulation was responsible for this observed secretory phenotype, and not the virus infection itself. The use of conditioned media represents the physiological mixture of cytokines that would be present during influenza infection, and allowed us to study the effects of cytokines independently from the virus.

Previous studies have shown that individual cytokines may have the capacity to modulate ion channel expression[48,107] in airway epithelium; therefore, we dosed cells with IL-8 in order to assess whether, or not, this particular pro-inflammatory cytokine would be able to reproduce the same effect as the conditioned media (Objective 4). We conclude from our data that a dose response of IL-8 was able to induce a secretory phenotype through increased calcium-activated

I_{sc}. However, the underlying mechanism driving this increase did not correlate directly with up-regulated levels of KCNN4, as seen at an MOI of 0.6 and treatment with conditioned media. Although the investigation into the mechanism driving this increase is beyond the scope of this study, it is still possible that IL-8 up-regulates a potassium channel other than KCNN4 or KCNQ1 in order to drive this response. Therefore, our hypothesis that IL-8 exposure would increase KCNN4 and KCNQ1 ion channel function was not confirmed; however other potassium channels may be involved. Together, these studies have revealed that the development of a secretory phenotype in influenza-infected airway epithelium is a result of the pro-inflammatory cytokine mixture acting in a paracrine manner on non-infected cells. These cytokines are capable of up-regulating KCNN4 and KCNQ1, which then increase the driving force for chloride secretion.

These potassium channels have not been considered as potential targets for the treatment of influenza-induced ARDS to date, but the results presented here indicate that this may be a viable option. KCNN4 already has a clinically available drug, ICA-17043, which has been used to successfully treat individuals with sickle cell anemia[139,140,148]. Given that the target is the same, this compound may prove to have dual purpose. Additionally, this research may have implications for other respiratory viruses as many complications associated with respiratory pathogens involve formation of pulmonary edema[155,156]. Respiratory syncytial virus (RSV) in particular has been shown to modulate vectorial ion transport in airway epithelium[155]. If the mechanisms are similar, the therapeutic benefits of potassium channel inhibitors may extend to treat multiple respiratory pathogens.

Overall, we conclude that the pro-inflammatory cytokines secreted by infected epithelial cells are capable of inducing a secretory response independently of the virus. The concerted up-regulation of KCNN4 and KCNQ1 after CM treatment indicates that both of these channels are required to increase apical chloride secretion. IL-8 treatment resulted in a similar increase in secretory phenotype; however, the underlying mechanism remains to be uncovered, as it is not a result of increased KCNN4 or KCNQ1. This study reveals the impact cytokine expression levels have on airway epithelial cell secretion, and strongly suggest that the host immune response drives significant components of influenza virus pathogenesis.

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